ROLE OF NLRP3-INFLAMMASOME IN FUNCTIONAL DECLINE IN PHYSIOLOGICAL AGING. Implications for cardio-metabolic events

Fabiola Marín Aguilar

ROLE OF NLRP3-INFLAMMASOME IN FUNCTIONAL DECLINE IN PHYSIOLOGICAL AGING.

Implications for cardio-metabolic events

Doctorado en Ciencias de la Salud

Memoria presentada por Dña. Fabiola Marín Aguilar para optar al grado de Doctora por la Universidad de Sevilla

Dr. **PEDRO BULLÓN FERNÁNDEZ**, Catedrático del Departamento de Estomatología de la Universidad de Sevilla

Dr. **MARIO DAVID CORDERO MORALES**, Investigador Posdoctoral del Departamento de Fisiología de la Universidad de Granada

CERTIFICAN:

Que la Tesis Doctoral titulada "**ROLE OF NLRP3-INFLAMMASOME IN FUNCTIONAL DECLINE IN PHYSIOLOGICAL AGING. Implications for cardio-metabolic events**" realizada por Dña. Fabiola Marín Aguilar para optar al grado de Doctor, ha sido llevada a cabo bajo nuestra dirección.

V.º B.º

Los Directores

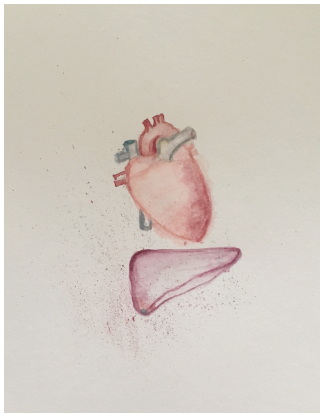
Dr. Pedro Bullón Fernández Dr. Mario David Cordero Morales

A Victoria, pasado, presente
y aspiraciones futuras.

“Ningún mar en calma hizo experto
a un marinero”

LIST OF CONTENTS

CHAPTER 01



General introduction

&

Thesis outline

Fabiola Marín-Aguilar, *et al.* Adenosine monophospate (AMP)-activated protein kinase: A new target for nutraceutical compunds. *International Journal of Molecular Sciences.* **2017**, 18: 288

Luis E. Pavillard*, Fabiola Marín-Aguilar*, Pedro Bullón, Mario D. Cordero. Cardiovascular diseases, NLRP3-inflammasome, and western dietary patterns. *Pharmacological Research.* **2018**, 131: 44- 50

The Science of Aging

1. Generalities

Why does aging occur? This is a common and tough question that arises as time goes by. Aging constitutes one of the biggest concerns for the human being and to ask why do we age is to enter the field of evolutionary biology, which is crucial to understand health and disease [1]. Nowadays, researchers' challenge focus on explaining the reasons of aging rather than the obvious drawbacks of the natural process.

Aging is commonly characterized as a progressive and generalized impairment of function, resulting in an increased vulnerability to genetic and environmental factors [2-4]. Getting older is in fact, an enigmatic biological process highly medically relevant, since despite considering increased longevity a remarkable achievement for the humankind, aging is the major risk factor for the development of chronic diseases and represents an extraordinary financial burden on the health care systems [5], therefore constituting a sanitary and a socioeconomic concern. It is estimated that in twelve years, a great percentage of the population will be aged 65 or older [6] **[Figure 1]**, and according to the World Health Organization (WHO), between 2015 and 2050, the proportion of the world's population over 60 years old, will nearly double, from 900 millions to 2000 million people, which will represent an increase of 10% [7].

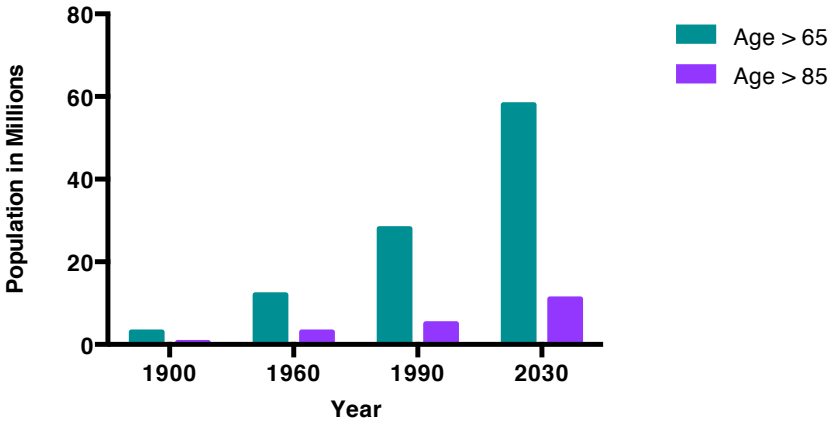


Figure 1. **Population becomes older**. People aged 65 or older (light bars) and 85 years old or older (dark bars) in the United States from 1900 through 2030. Data and image credit: US Census Bureau data with projections for 2030 and, Lakkata EG, Levy D. Arterial and cardiac aging: Major shareholders in cardiovascular disease enterprises, part I: aging arteries: a "set up" for vascular disease. *Circulation*. **2003**, 107: 139-146.

The aging process is linked to an accumulation of mutations and genomic instability resulting in a progressive functional and structural decline in multiple organs. Then, far from being considered an illness in itself, aging is the greatest risk factor for the onset of chronic age-related diseases including cardiovascular disease, cancer, diabetes, neurodegenerative diseases, and higher tendency to infection [8].

As aforementioned, accumulation of DNA damage is a common feature of aging cells that generally occurs during DNA replication step, where most cells undergo division in order to maintain cell population in tissues and thus, a proper function of the organs. It is crucial for cells to accurately replicate their DNA, since during copying DNA strands, mistakes may occur and such inaccuracy may introduce alterations in cellular function. Although cells have evolved a complex network of DNA-repair mechanisms that are collectively capable to deal with most

of the DNA damage affecting the nuclear DNA, these mechanisms may not be perfect and mistakes can sleep through. It has been shown in many studies that such mistakes in DNA accumulate in cells with age [9-12]. Thus, it is vital to underline that the mutations introduced during DNA replication that remain after unsuccessful repair mechanisms are irreversible, causing genetic damage accumulation and increasing genomic instability during lifetime.

1.1. Cellular Aging and the Senescence-Associated Secretory Phenotype

Cellular or replicative senescence was first proposed in 1961 [13, 14]. Since that time, it has been shown in various studies to be a significant tumor restricting mechanism that halts proliferation in response to damage that occurs during replication [15-19], enabling the organism to antagonize the potentially detrimental effects of uncontrolled growth. Morphologically, senescence cells acquire a characteristic morphological type consisting on cellular enlargement and flattering. However, senescent cells can also acquire a secretory phenotype, which is damaging and irreversible, termed senescence-associated secretory phenotype (SASP). This deleterious phenotype involves secretion of interleukins and growth factors, degradative enzymes like metalloproteases, and insoluble proteins [15, 20] that may alter tissue homeostasis and affect neighbouring cells promoting tumour progression [21-23].

The senescent phenotype has been widely associated with driving organismal aging. The SASP is a response to genomic damage that stimulates tissue repair by attracting immune cells and subsequently, induce local inflammation. What it is very interesting, is the belief that SASP also has certain beneficial effects by secreting

several chemokines and cytokines that activate immune cells to specifically target and clear senescent cells from tissues [24, 25]. Besides, some SASP factors are also involved in tumour suppressive growth arrest of cells [26, 27]. However, aging determines the change from a temporal programmed process to a continual stochastic response, since there is an evident age-related decline in the immune system and in the efficiency of senescence-cell clearance.

SASP factors, because of cellular senescence, are involved in several chronic processes **[Figure 2]**. Recent research performed in murine models report that the onset of chronic diseases is delayed when senescent cells are genetically removed [28]. Byun et al., [29] definitively proposed the connection between cell senescence and age-associated diseases where they postulate that senescent cells have the ability to modulate young cells by releasing inflammatory SASP factors, such as IL-1. The SASP enables the onset of different pathologies by promoting a proinflammatory state. For instance, obesity, type 2 diabetes mellitus, hypertension and atherosclerosis, among other cardiovascular risk factors that compromise metabolism, are associated with cellular senescence and SASP [30].

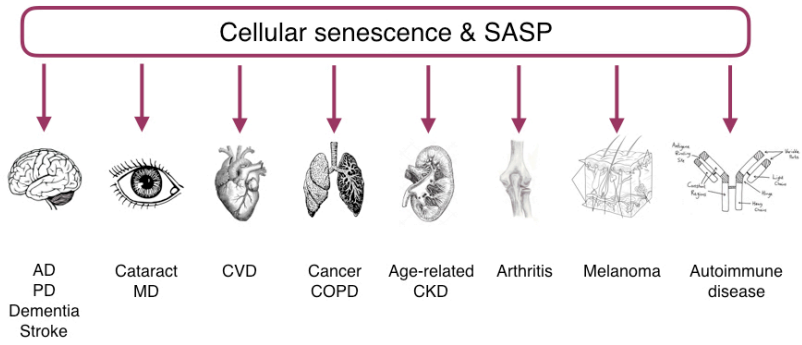


Figure 2. Illustrative representation of tissues and their related diseases as we age. Both cellular aging and senescence-associated secretory phenotype (SASP) are involved in the onset and development of inflammaging or aged-related pathologies linked to inflammation. Abbreviations: AD- Alzheimer's disease; PD- Parkinson's disease; MD- Macular degeneration; CVD- Cardiovascular disease; COPD- Chronic obstructive pulmonary disease; CKD- Chronic kidney disease.

1.2. Aging theories: Evolutionary and molecular basis outlook

The complexity of the aging process can be elucidated by many theories. However, there is not yet an exact mechanism to explain how this phenomenon occurs. In 1882, August Weismann proposed the wear and tear theory. The basis of this theory, consist on comparing organisms to machines, and holds that cells, like components of an aging car, burn out with time from repeated use and accumulate damage due to an excessive consumption of fat, sugar or, exposure to UV radiation, finally leading to a functional decline in organs [31]. However, this theory does not take into account that cells are able to repair from DNA damage. Consequently, the stochastic theory arose as a byproduct of this hypothesis suggesting that aging is due to an inefficiency of cell mechanisms to repair damage caused by environmental stress, UV radiation, or infections [32].

Theories of aging regarding evolution propose the idea that certain genes involved in longevity are also involved in the homeostasis of somatic cells, and very prone to mutations during aging.

Two of the main evolutionary theories of aging are possibly Medawar's mutation and accumulation theory (Medawar 1952) [33] and the antagonistic pleiotropy theory (Wiliams 1957) [34]. Both theories hold a concept based on the same principle: As we age, natural selection is not effective enough to eliminate deleterious genetic variations, whose effects will be shown in late periods in life.

In this context, Medawar proposes that genomic variations associated with certain diseases, or with physiological decline in aging, tend to accumulate and take place once reproductive years are gone. On the other hand, Williams postulates the antagonistic pleiotropy theory, adding an adaptive aspect to the first proposed hypothesis: genetic variations linked to age related diseases may be conserved in the population if these variations confer an adaptive advantage during reproductive years, either protecting against an illness, or increasing reproductive possibilities. The mutation accumulation and the antagonistic pleiotropic theories provide together, the basis for much of the ongoing thinking about the evolutionary genetics in the field of aging [35, 36].

At a molecular level, evidence suggests that most of the main mechanisms involving damage are linked to macromolecules. The main theories that explain how aging occurs are illustrated here below:

1.2.1. Somatic DNA damage and Mutation Theory

Since 1994, it is believed that there is a general and solid relationship between longevity and DNA repair [37], suggesting that the capacity for DNA repair is an important aspect to determine the rate of aging at cellular and molecular levels. Studies on the enzyme poly (ADP-ribose) polymerase-1, also termed PARP-1, can clarify this fact since this molecule is a key player in the immediate cellular response to stress induced DNA damage [38]. Higher expression of PARP-1 activity level is associated with longer life spans between species and within species [39, 40].

1.2.2. Mitochondrial Decline Theory and Oxidative Stress Theory

Apart from nuclear DNA, certain mutations can also cause damage in mitochondrial DNA (mtDNA) as we age [41]. It has been reported that there is a link between molecular stress and aging caused by the accumulation of mtDNA mutations. Cytochrome C oxidase (COX) deficient cells are directly associated with mtDNA mutations in human muscle [42, 43], brain [44, 45] and gut [46].

One of the best-known and most conventional approaches to date is the mitochondrial free radical theory of aging or the nowadays-termed oxidative stress theory (OST) [47]. Oxidative stress hypothesis interprets aging at a molecular level, explaining that aging occurs due to an imbalance between the production of reactive oxygen species (ROS) and the capacity of the biological system to repair the outcoming damage, resulting in the failure to maintain the mitochondrial integrity and DNA repair. ROS molecules include superoxide ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen

dioxide ($NO_2^{\bullet}$), and peroxyl ($ROO^{\bullet}$), produced either as by-products during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes.

1.2.3. Telomere Shortening Theory

Telomeres are repeated nucleotides sequences that are believed to protect the ends of linear eukaryotic chromosomes from fusing other DNA strands. During cell division, telomeres tend to get progressively shorter, losing a little bit of their length [48]. Since telomerase, which is a specialized DNA polymerase that could replicate telomeres, is only present in germ cells (testis and ovary) and is absent in normal mammalian somatic cells, telomeres become too short to replicate showing a decline in cellular capacity division with age.

Eventually, the cell will arrest the cycle and enter cellular senescence. Some suggest that in dividing somatic cells, after a fixed number of divisions, telomeres protect us against uncontrolled cell replication as happens in cancer, but causing aging as a side effect [49]. It has been reported that telomeres are particularly susceptible to accumulation of DNA damage with age, and this damage is restricted to be repaired because of a multiprotein complex termed shelterin that binds to the telomeres and prevents the access of DNA repairing proteins to the telomeres. Therefore, DNA damage at telomerases is persistent, inducing cellular senescence [50].

1.2.4. Altered Proteins and Waste Accumulation Theory

Protein turnover is vital to maintain cell function by removing damaged or unnecessary proteins. Chaperones help sequestering and restoring denatured proteins and proteasomes. Proteasomes are involved in recognizing and selectively degrade damaged and ubiquitinated proteins. When there is not a correct recycling process and damaged proteins accumulate with time due to impairment in protein turnover, there is a great tendency to develop a wide range of age-related pathologies, including cataract, Alzheimer's, and Parkinson's disease. There is evidence suggesting that exits a functional decline in the activities of both, chaperones [51] and proteasomes [52] as we age. These declines are translated into a more general and serious failure caused by waste accumulation [53].

Until date, several aging mechanisms are widely acknowledged. However, they are not fully satisfactory. Most research is focused on unique mechanisms of theories that indicate that hypothetical molecular and cellular lesions do occur as we age, but there is no data evidencing the theory itself to be sufficient to clarify age-related diseases. Much of the recent proliferation of aging theories arose from an inquisitiveness to see the different hypothesis competing to better explain how aging occurs. Therefore, recent initiatives state that these theories may interact with each other in a more sophisticated manner, like a network of aging theories considering the contribution of various mechanisms together and allowing the interaction among different processes [54].

For instance, it is well known that a gradual accumulation of mutations in mtDNA over years, may lead to a steady increase in both ROS production, and energy consumption. This initiates the process,

but what ultimately damages the cell is the collapse generated in the homeostatic mechanism. By understanding these connections, it may be possible to mediate against cell deterioration and promote successful aging [3].

Advancing age is the major risk factor for the development of many chronic diseases, and more specifically, for cardiovascular disease (CVD), which has become a widespread global fact no longer considered a disease restricted to western-type societies. Cardiovascular events represent more than 30% of all deaths worldwide, including hypertension, myocardial infarction, and stroke [55]. Many of these events occur prematurely before the age of 60, and according to the World Health Organisation (WHO), the majority of them could have been prevented [56].

2. Aging and Calorie Restriction

Cardiovascular disease (CVD) is traditionally believed to be due to an accumulation of cholesterol and fatty acids in the heart and vessels, triggering an inflammatory response and ROS production, and therefore compromising tissue function. A different point of view arose in 1935, when McCay et al., reported that dietary or caloric restriction (CR), a 20 to 40% reduction in calorie intake, definitely extended lifespan in rats [57]. Here emerged a new concept in the aging field stating that a high-calorie diet without exercise may supress the expression of genes involved in longevity, since it has been reported that CR reduces insulin-like growth factor-1 (IGF-1) levels in yeast and mammals, and prevents the onset and development of age-related cardiovascular events [58-60]. For example, CR delays the development of atherosclerotic lesions in mice [61], improves LV diastolic function of the aging heart [62, 63] and diminishes arterial

stiffness [59, 64]. Thus, long-term CR has a potent effect in preventing and delaying severe cardiomyopathy in various animal models.

Interestingly, some exceptions and adverse results in certain inbred and outbred strains of mice have been reported [65-67], suggesting that CR on survival is not universal and may affect health and lifespan depending on the strain and sex [68]. However, data from human studies indicate that populations like Okinawans, who intake a slightly reduced calorie diet (1785 kcal/d approximately), show lower rates of coronary heart disease and higher frequency of centenarians. According to this fact, some studies report that individuals under long-term calorie restriction are less prone to develop cardiovascular events [69].

CR plays a role in the modulation of longevity pathways, since CR regulates four key genes involved in aging: AMP-activated protein kinase (AMPK), insulin/insulin-like growth factor 1 (IGF-1), nicotinamide adenine dinucleotide $(NAD)^{+}$-dependent deacetylases (sirtuins), and the mammalian target of rapamycin (mTOR). Consistent with this, there is reasonable interest in finding pharmacological agents that mimics CR. Resveratrol [70], rapamycin [71] and more recently, metformin [72, 73] are some examples of the main compounds that mimic CR and prevent pathology onset by regulating genes involved in longevity pathways **[Figure 3]**. Some examples of a variety of model organisms are: the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the fly *Drosophila melanogaster*, which have served to identify a number of longevity genes, their related pathways and therefore, their connection with aging and more specifically, with their roles in cardiovascular aging.

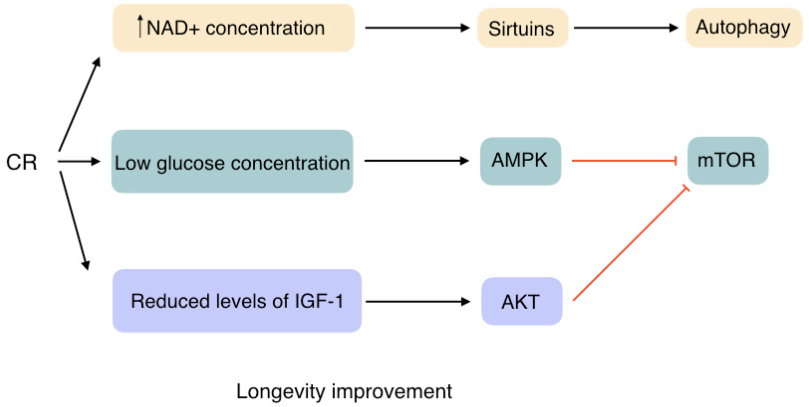


Figure 3: Calorie restriction promotes improved longevity. By modulating certain factors involved in the longevity network, caloric restriction may enhance not only lifespan, but also health-span. Black arrows indicate activation and red arrows indicate inhibition. Abbreviations: CR- Caloric restriction, NAD- Nicotinamide adenine dinucleotide, AMPK- Adenosine monophosphate protein kinase, mTOR-mammalian target of rapamycin, IGF-1- Insulin-like growth factor-1, AKT- Serine/threonine kinase Akt, also designed protein kinase b.

3. Major genes involved in Aging

Four main important genes including AMPK, IGF-1, Sirtuins, and TOR govern longevity pathways.

3.1. AMP-activated protein kinase (AMPK)

AMPK is composed of two α, two β and three γ subunits [74], which are regulated by AMP binding. AMPK works as a metabolic sensor regulating the AMP: ATP ratio and is involved in glucose and lipid metabolism. In heart tissue, AMPK activity plays a role in fatty acid and glucose uptake and glycolysis, whereas in liver, is involved in fatty acid and cholesterol synthesis and glucogenesis [72, 75]. It modulates autophagy and mitochondrial function [76] by triggering the destruction

of defective mitochondria through a mitochondrial-specific process of autophagy termed mitophagy and through activation of mitochondrial biogenesis. With concern to longevity network **[Figure 4]**, AMPK modulates some important pathways, including IGF-1 pathway through the extracellular signal-regulated kinase (ERK1/2) cascade [77], modulates the abundance of NAD and nicotinamide phosphoribosyltransferase (Nampt) [78, 79], and inhibits mTOR through direct phosphorylation of the TSC1/2 complex [6]. AMPK is essential to maintain cardio-metabolic homeostasis. This kinase has shown to be cardioprotective during ischemia. According to some research, mouse models lacking AMPK functionality, had exarcebated myocardial infarction [80]. Activation of AMPK by metformin reduced overload-induced cardiac hypertrophy [81] and thirdly, mutations in the regulatory γ 2 subunit led to a familial syndrome of hypertrophic cardiomyopathy [82]. Considering that AMPK is a master regulator of energy balance and metabolism, its modulation has shown to be an interesting target to regulate metabolic pathways. Interestingly, it has been recently reported that nutraceutical compounds, including resveratrol, hydroxytyrosol or some flavonols among others, have AMPK-mediated therapeutic effects on diabetes mellitus and CVD [74], which gives a natural approach to AMPK pathway activation.

3.2. Insulin/insulin-like growth factor 1 (IGF-1)

One of the first genes to be characterized as a longevity gene was IGF- 1. The loss of IGF- 1 in the nematode *C elegans* showed to increase lifespan [83] and, decades of studies pointed to reduced IGF-1 signaling as a strategy to prevent cells from aging and pathology onset. These findings led to identify all the genes that could be involved in IGF-1 pathway to modulate lifespan. In the longevity network **[Figure 4]**, IGF-1 signaling modulates mTOR through Akt activity [84]. At heart

level, overexpression of IGF-1 showed to protect against myocardial cell death after infarction and reduced hypertrophy and diabetic cardiomyopathy [85, 86]. Paradoxically, other studies report that overexpression of IGF-1 led to cardiac hypertrophy and heart failure [87]. These variations may be due to existing different isoforms of IGF-1.

3.3. Sirtuins (Sir2)

The sirtuins are a family of proteins that function as nicotinamide adenine dinucleotide (NAD)-dependent deacetylases and ADP-ribosyltrasnferases. In mammals, seven sirtuin family members exist, and have different localizations in the cell. For example, Sirt3, Sirt4, and Sirt5 are found exclusively within the mitochondria. However, Sirt1 and Sirt6 are generally localized in the nucleus. Sirtuins regulate several cellular functions including DNA repair and cell cycle among others [88]. The role of sirtuins was first observed in a model of replicative lifespan in yeast, which measured the number of times a yeast mother cell produced a daughter cell before going senescent. Up until now, numerous studies have kept sirtuins as a key component of the longevity network **[Figure 4]**, having shown to modulate aging from yeast to mammals [89-93]. For instance, Sirt1 regulates AMPK pathway through deacetylation of liver kinase B1 (LKB1) [94], regulates IGF-1 through modulation of UCP2 expression [95], and interacts with TSC 1/ 2 by inhibiting mTOR activity [96, 97]. Within cardiovascular aging and disease, some studies report that Sirt1 knock out (KO) mice have greater injury in response to ischemia reperfusion, which is attenuated in Sirt1 transgenic mice [98]. Cardiac-specific Sirt1-overexpressing mice showed delayed age-related cardiomyopathies and resistance to oxidative stress and apoptosis. Interestingly, when extensive Sirt1 overexpression occurred (around 20 fold), resulted in undesirable

effects including oxidative stress, apoptosis and cardiomyopathy [99, 100]. Mitochondria-localized Sirt3 showed to prevent age-dependent cardiac dysfunctions in KO mice [101, 102]. Mice deficient in Sirt6 developed a drastic premature aging phenotype, characterized by a failure to grow healthily, intestinal sloughing, hypoglycaemia and remarkable shortened lifespan [90]. Finally, Sirt7 seemed to protect mice from cardiac hypertrophy and inflammatory cardiomyopathy [93]. Despite the fact that research reports that sirtuins seem to have many beneficial effects on cardiovascular health, the molecular mechanisms by which sirtuins regulate the heart is not yet fully understood.

3.4. Mammalian Target of Rapamycin (mTOR)

The serine- threonine protein kinase mammalian target of rapamycin (mTOR) is a master regulator of cell growth and metabolism. Deregulation of mTOR pathway may lead to human diseases, such as cancer [103], diabetes, obesity and neurological and genetic alterations [104]. Deletion of TOR or its inhibition through treatmeant with rapamycin has been observed to extend lifespan in yeast, flies, worms and mammals [71, 105- 107]. Inhibition of mTOR signalling in the heart with rapamycin restrains cardiac hypertrophy caused by pressure overload [108, 109], probably through mTOR control of protein translation and cell size blockage. Moreover, the PI3K/AKT/mTOR pathway in which is involved plays a main role in a number of signalling pathways, placing this pathway as a crucial mediator of cardiovascular metabolism and aging. Regarding the longevity network **[Figure 4]**, mTOR acts as an effector of upstream sirtuin/AMPK/IGF-1 activity and its inhibition, either through rapamycin or nutrient-poor conditions activates autophagy, which promotes cardiovascular health by destructing defective macromolecules [110].

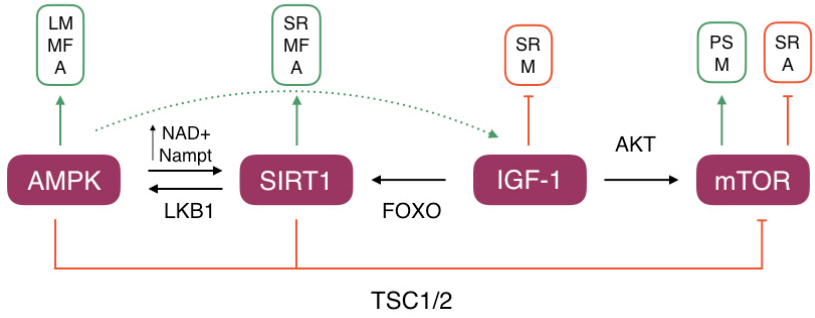


Figure 4: Longevity network organization. Major genes involved in longevity network system concerning aging and lifespan. Abbreviations: LM- Lipid metabolism; MF- Mitochondrial function; A- Autophagy; SR- Stress resistance; PS- Protein synthesis; M- Metabolism. Green and black arrows indicate activation of a physiological function or a concrete gene, respectively. Red arrows indicate their inhibition.

In a recent study, López- Otín el al., have explained aging decline as a result of a number of different events occurring in the organism that can be described and classified into several “hallmarks” [50]. Among these hallmarks of aging are included: loss of proteostasis, epigenetic alterations, telomere shortening, DNA damage or genomic instability, deregulated nutrient sensing, accumulation of dysfunctional organelles, cellular senescence, stem cell exhaustion and altered intracellular communication. Furthermore, the self-degradative process of autophagy, which is crucial to maintain cardiac homeostasis and confers the cells the ability to adapt to unfavourable environments and stress **[Figure 5]**, is unquestionably a main contributor to progressive decline in aging and loss of cell quality control.

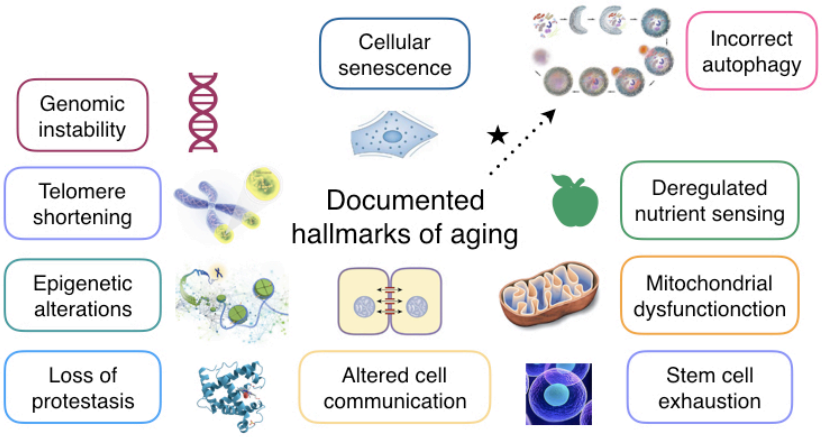


Figure 5: Correct autophagy is essential to promote healthy aging. Documented hallmarks of aging by López- Otín et al., including inaccurate autophagy as a marker involved in aging decline.

4. Autophagy and aging

Autophagy is a great cellular quality control pathway, responsible for selectively removing dysfunctional organelles and protein aggregates to promote cell survival. It is generally accepted that aging is characterized by a reduction in autophagy activity [111-116], believing that insufficient autophagy is a major contributor to aging decline.

Autophagy is a tightly regulated degradation pathway, whose activity is regulated by the coordination of both intracellular and extracellular signals, being AMPK, mTOR and complex III PI3K central regulators of the process. mTOR is involved in cell growth and metabolism. When it is activated, autophagy gets inhibited. For example, in a situation of nutrient enrichment environment, the PI3K/AKT signaling pathway, specifically inhibits autophagy through activation of mTORC1 complex [115-117]. Contrarily, under nutrient

limiting conditions autophagy becomes active by AMPK, via various mechanisms. For example, AMPK activates autophagy by inhibiting mTORC1 **[Figure 4]** through phosphorylation of RAPTOR [118] or by activating TSC1/2 [119]. AMPK is also involved in autophagy activation through Unc-51 like kinase-1 (ULK1) [120] and Beclin-1 complex [121].

Autophagy consists therefore, on several sequential steps. Autophagy-related proteins (ATG) regulate the biogenesis of the autophagosome. The complex in charge of the initiation of the phagophore formation is promoted by the activation of the core proteins Beclin1-Vps34-Vps15 through ULK1 [122]. Then, the elongation of the phagophore into an autophagosome involves two ubiquitin-like conjugation systems, Atg5-Atg12-Atg16L and microtubule-associated protein 1 light chain 3 (LC3) [123]. The resulting conjugated LC3 II is then integrated into the membrane and is necessary for pagophore elongation and cargo recognition [124]. The phagophore continues growing until it closes on itself, absorbing the cargo to be degraded. A cargo-loaded autophagosome is formed to fuse with a lysosome and originate an autolysosome. Finally, the generated cargo and the inner membrane of the autophagosome are degraded by the lysosomal hydrolases obtaining basic components to promote cell survival [125].

At mitochondrial level, also exists a form of autophagy, which consists on cleaning dysfunctional mitochondria due to damage or stress [126]. This process is termed mitophagy and examples of proteins involved in mitophagy include p62/Sqstm1, or PINK/Parkin pathway, responsible for marking depolarized mitochondria [127]. Premature aging phenotype and reduced lifespan have been associated with mice lacking the protein p62/Sqstm1 [128]. This lack may lead to accumulation of dysfunctional mitochondria in cardiac tissue, what suggests that autophagy plays a vital role in preventing premature

aging.

Furthermore, studies have reported that genetic or pharmacologic improvements in autophagy should counteract age-associated decline leading to extended lifespan [129-133], whereas inhibiting autophagy might shorten lifespan [134]. For instance, pharmacological activation of autophagy using rapamicyn, the mTOR inhibitor, promotes longevity in the fly *Drosophila melanogaster* [135] and in aged mice [71, 136]. Likewise, mice with reduced mTOR expression have shown longer lifespan [137].

Manipulation of autophagy also affects cardiac function and health. It has been demonstrated that long-term intake of fat inhibits autophagic flux in heart in an animal model [138]. CR however, which is a potent inducer of autophagy in heart, produces the opposite effect [139]. Long-term CR treatment is linked to AMPK activation, and preserved contractile function in aged mice [131]. Moreover, administration of rapamycin showed to reverse age-related decline in cardiac function in mice [140].

Despite the fact that several studies report that autophagy is reduced in aged tissues [111-115], studies on autophagy in the aging heart are inconsistent. Some report that autophagy is increased; some say that it is decreased and in other situations, autophagy remains unchanged. On the one hand, it has been reported that aged mice hearts show higher levels of Beclin1 and LC3 II/I [141], on the other hand, an increase in LC3 II levels was observed in aged tissues, but no changes were shown in Beclin1 or p62 levels [142]. Surprisingly, another study reports a decrease in LC3 II levels in murine cardiac tissue [134].

Gathering all these data, one gets to the conclusion that such differences may be due to experimental design or to strain and background of mice. What it is clear is that additional research is needed to better understand autophagy in cardiac tissue and therefore, cardiac aging.

Until date, many theories and biological processes could explain aging decline and how it constitutes a major risk factor for cardiovascular and cardio-metabolic events. Nevertheless, several studies demonstrate that immunological inflammation might be highly associated with aging, since the immune system is programmed to decline over time [143]. This concept is termed immunosenescence, where the innate immunity response is remarkably activated, leading to the senescent low-level, chronic inflammatory phenotype known as inflammaging.

5. The NLRP3-inflammasome and inflammaging

The inflammasomes are intracellular multimeric complexes that drive the activation of inflammatory caspases [144]. In particular, NLRP3 inflammasome has been involved in the pathogenesis of several acquired inflammatory diseases, such as Cryopyrin-associated periodic fever syndromes (CAPS) caused by inherited NLRP3 mutations [145]. NLRP3-inflammasome is also involved in metabolic-induced complications characterized by a low-grade systemic inflammatory state triggering an innate immune response to a multitud of endogenous metabolic danger patterns inducing sterile inflammation. In that respect, NLRP3-inflammasome complex has been described to be activated when high-fat, and high-sugar nutrients are consumed, contributing to several inflammatory and metabolic disabilities onset [146]. Overnutrition and acquired western-type habits are described as major

risk factors for cardiovascular and metabolic events onset [147].

Therein, all these metabolic-induced complications and age-reated diseases share a low-grade systemic inflammatory response that triggers NLRP3 activation. In aging field, this typical inflammatory condition is termed inflammaging.

The idea of inflammaging was originally introduced in 2000 [148] by C. Franceschi, referring to a progressive increase of a proinflammatory state, a systemic low-grade inflammatory process that contributes to the onset and development of chronic diseases and degenerative changes as we age. Curiously, age-related inflammation in certain organs may lead to a noticeable decline even in the absent of any disease. For instance, chronic inflammation is at the same time related to aging and also to its associated diseases, including diabetes, atherosclerosis, cancer and neurodegenerative diseases. It is generally believed that systemic low-grade chronic inflammation potentially contributes to incremented age-associated disease prevalence and severity [149], since aging is related to an increase in interlukins, such as IL-18, IL1β, and IL-6. Furthermore, IL1β and IL18 are particularly produced after inflammasome-dependent caspase-1 activation and interestingly, the majority of the endogenous signals that have been described as inflammasome activators, are known to accumulate as we age.

5.1. Structure of NLRP3-inflammasome

Structurally, inflammasomes consist of an intracellular sensor protein, the Nod-like receptor (NLR); the adaptor apoptosis- associated speck-like protein containing a caspase recruitment domain (ASC) and, the proinflammatory caspase-1 precursor [150]. NLRs are classified

according to the domain structure. Generally all NLRs, except for NLRP10, contain a leucine-rich repeat (LRR) domain, which is believed to provide the critical structural framework for molecular interactions [151], and enables the recruitment of caspase-1, either directly through CARD-CARD interactions, such as NLRC4 inflammasome, [152] or indirectly through a PYRIN domain that is shown to bind to apoptosis-associated speck-like protein containing a CARD (ASC).

Apart from the NLR family, non-NLR proteins such as the AIM2-like receptor (ALR) family can also assemble to conform inflammasomes. ALR family contains an HIN200 DNA-binding domain instead of an LRR. Therefore upon sensing certain stimuli, either NLR or AIM2 can oligomerize to be a caspase-1 activated scaffold.

Interestingly, NLRs share a similar structure which consist of three domains:

1. NOD or NACHT, a central nucleotide binding oligomerization domain.

2. A C-terminal domain, LRR, which is present in all inflammasome members except for NLRP10.

3. An N-terminal domain of recruitment of effector molecules, which determines the classification of the different NLRs **[Figure 6]**.

NLPR3 is probably the most in depth-studied inflammasome due to its gamut of known stimuli activating the complex.

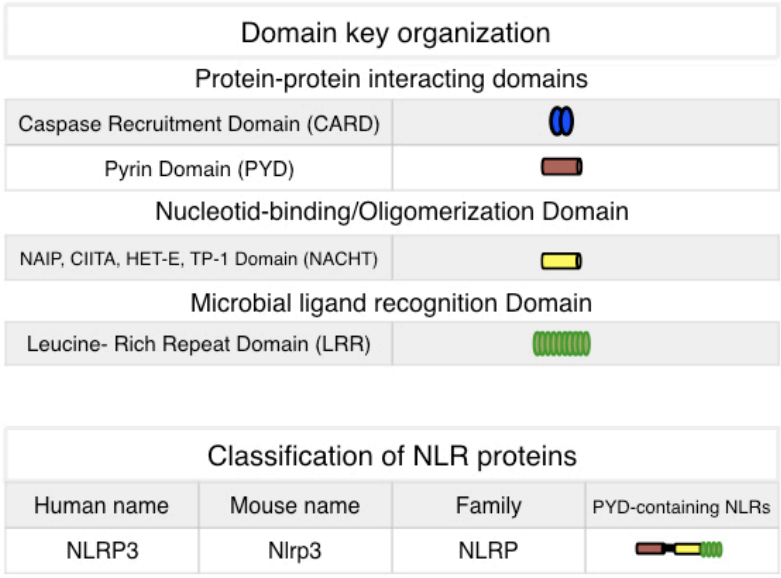


Figure 6: Domain key organization and NLRP3-inflammasome classification according to its domain structure. Inflammasomes are named according to the protein forming the scaffold. NLRs proteins are categorized in four subfamilies depending on the kind of N-terminal domain: N-terminal domain in NLRP subfamily is PYD.

Despite the fact that Nlrp3 inflammasome is the most thoroughly studied NLR, its complex mechanism of activation has shown to be even more sophisticated with the discovery of non-canonical inflammasome activation [153]. Here, the authors show that caspase-11 is critical for caspase-1 activation and IL-1β production in C57BL/6 Casp11 gene-targeted mice macrophages infected with *Escherichia coli*, *Citrobacter rodentium* or *Vibrio cholerae*.

5.2. Mechanisms of activation of NLRP3-inflammasome

Schroder and Tschopp [150] first described the inflammasome as a complex of multimeric proteins that assemble in the cytosol when pathogenic microorganisms or sterile stressors are present, considering this large complex tightly linked to the onset of the inflammatory

response, since they act as intracellular innate immune sensors [150]. Among all the inflammasomes described until date, the most in depth studied is the NLRP3-inflammasome, presumably because of its complete range of activators. NLRP3 can be activated in response to many different microbial, environmental or metabolic stimuli, which conclude in ROS generation, changes in ion flux, or cathepsin B emanation into the cytoplasm [154-156] **[Figure 7]**.

5.2.1. Canonical activation of NLRP3-inflammasome

Although under physiological conditions NLRP3 remains inactive, the complex requires two steps to be fully activated **[Figure 7]**. The first signal or priming consists of the activation of nuclear factor κB (NF-κB) [157] and the transcription of inflammasome constituents [158]. Signal two or triggering is represented by sensing a gamut of pathogen-associated molecular patterns (PAMPs) and stress-associated signals or host-derived damage-associated molecular patterns (DAMPs) to trigger NLRP3 oligomerization and final complex assembly [158-160]. The inflammasome assemblage concludes with the activation of caspase-1, which generates a signaling cascade that leads to maturation and pro-inflammatory cytokines (IL-1β and IL-18) release. Active caspase-1 also promotes pyroptosis in an independent manner of IL-1β maturation [152, 161]. Pyroptosis is a key defense mechanism against microbial infections, which acts blocking intracellular pathogens replication and promoting phagocytosis of surviving microbes [162]. Interestingly, most of the DAMPs that provoke the inflammasome activation such as asbestos, silica [163], certain toxins, ATP [164] or uric acid crystals [165] among others, have shown to increase during aging [166].

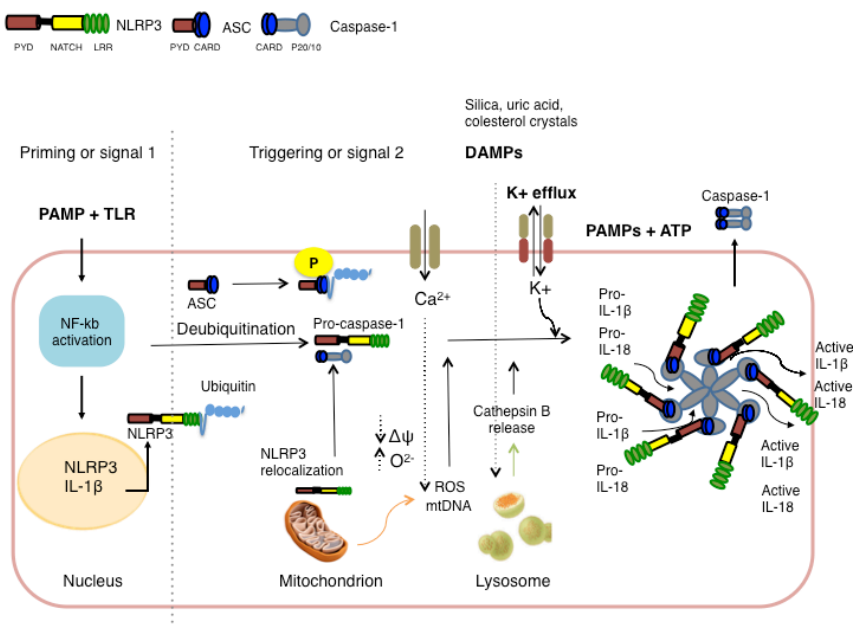


Figure 7: Canonical mechanism of activation of NLRP3-inflammasome. Activation of NLRP3-inflammasome requires two different signals: Signal 1 or priming and signal 2 or triggering. The fully activation of NLRP3 ends with caspase-1 activation and a form of inflammatory cell death termed pyroptosis. Abbreviations: PAMPs-Pathogen-associated molecular patterns, DAMPs-Damage-associated molecular patterns, TLR-Toll like receptor, NF-kb-Nuclear factor kappa beta, CARD-Caspase recruitment domain, and ASC-Apoptosis-associated speck-like protein containing a CARD.

5.2.2. Non-canonical activation of NLRP3-inflammasome

Appart from canonical activation, non-canonical caspase-11-dependent NLRP3 activation has been described as well [167]. Caspase-11 becomes active in response to cytosolic LPS gram-negative bacteria, including *Escherichia coli, Citrobacter rodentium,* and *Vibrio cholerae* [153], flagellin-deficient *Legionella pneumophila* [168], and *Salmonella typhimurium* [169]. Some studies suggest that TLR4-TRIF-mediated type I interferon dependent production of caspase-11 [170, 171] is required for non-canonical NLRP3-inflammasome

activation **[Figure 8]**. Once gram-negative pathogenes activate TLR4-TRIF pathways, nuclear translocation of NF-kb promotes the transcription of proinflammatory interleukins IL-1b and IL-18 on the one hand, and the transcription of interferon regulatory factors IRF3 and IRF7 genes, on the other [172]. Later, the IRF3–IRF7 complex provokes the expression of interferon-α/β, which binds to the corresponding IFNAR1/IFNAR2 receptor, finally driving to the JAK/STAT pathway activation and resultant transcription of caspase-11 gene [172]. Moreover, the binding of lipopolysaccharide (LPS) from gram-negative bacteria to caspase-11 has demonstrated to induce escaping phagosomes that activate caspase-11 [173, 174]. Then, activated caspase-11 activates pyroptosis through cleavage of gasdermin D, and promotes IL-1β release through activation of the NLRP3-ASC-caspase-1 pathway [175, 176] **[Figure 8]**.

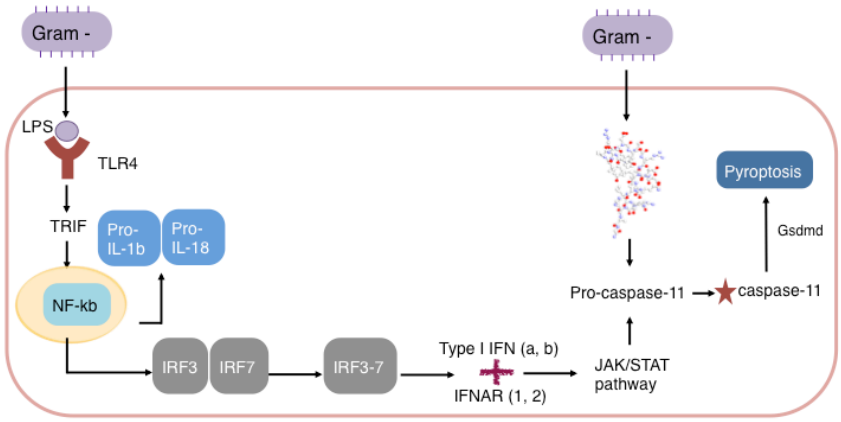


Figure 8: Non-canonical activation of NLRP3-inflammasome. Gram-negative bacteria LPS stimulate TLR4-TRIF pathways. Then, translocation of NF-kb promotes the transcription of proinflammatory interleukins on one sense and IRF3 and IRF7 genes transcription, on the other. Subsequently, the IRF3-IRF7 complex evoke the expression of type I IFN, which directly binds to its receptor, IFNAR, triggering the JAK/STAT pathway activation and caspase-11 gene transcription. Secondly, unknown scaffolding proteins induced by gram-negative bacteria cleave and stimulate caspase-11, which is the final responsible for the induction of pyroptosis and the NLRP3-ASC-caspase-1 pathway activation. Abbreviations: LPS- Lipopolysaccharide, TLR4- Toll like receptor 4, TRIF- Tir domain containing-adapter-inducing interferon beta, IRF- Interferon regulatory transcription factor, IFN- Interferon, IFNAR- Interferon alpha/beta receptor, JAK/STAT- Janus kinase/ signal transducer and activator of transcription, Gsdmd- Gasdermin D.

Both canonical and non-canonical ways of NLRP3-inflammasome activation take place independently. Nevertheless, it is interesting that caspase-11 has demonstrated to improve the canonical caspase-1 activation and IL-1b and IL-18 release in response to certain stimuli [165, 175]. In this context, further experiments should be performed to clear up the molecular mechanisms underlying the interaction between caspase-1 and -11 in promoting the canonical and/or non-canonical NLRP3 inflammasome activation.

5.2.3. Other NLRP3-inflammasome activators

Recently, several authors have reported some other proteins as activators of NLRP3-inflammasome as well [177-181], such as double-stranded RNA-dependent protein kinase (PKR), guanylate-binding protein 5 (GBP5), and NIMA-related kinase 7 (Nek7).

PKR has been reported to directly interact with almost all the known inflammasomes [178]. When PKR is removed, stimulation of caspase-1 and maturation of IL-1β and IL-18 is decreased in response to a range of stimuli. Nevertheless, additional investigations are needed to clarify the exact mechanism by which PKR is involved in NLRP3-inflammasome activation. It is also believed that GBP5 has shown to promote the activation of NLRP3-inflammasome in response to ATP, nigericin and certain bacteria [177], although how the protein stimulates the mechanism is still unknown as well.

Otherwise, recent data from various separate studies have shown the essential function of Nek7 in NLRP3-inflammasome activation [179-181]. Nek7 is part of the NIMA (never in mitosis gene a)-associated expressed kinase family, which regulates the mitotic development and DNA damage response [182] and seems to play a vital role in development and survival: a study performed in mice lacking Nek7 showed mortality either in late embryogenesis or in early post-natal period and severe growth delay [183]. Moreover, it has also been demonstrated that Nek7 is necessary for NLRP3-inflammasome activation by certain DAMPs including ATP, nigericin, monosodium urate (MSU) cholesterol crystals and alum [179, 180]. The interaction between Nek7 and NLRP3 is through the catalytic domain of Nek7 and the leucin-rich repeat (LRR) domain of NLRP3. Nek7 has recently shown to develop key roles in the regulation of NLRP3-inflammasome, including inflammasome oligomerization, ASC speck formation, and caspase-1 activation downstream of potassium efflux [180]. Nek7 is

necessary for NLRP3 activation in macrophages holding the NLRP3-activating mutation (NLRP3 R258W) [180]. Therefore, Nek7 has an essential role in NLRP3 activation. Three different in vivo models have confirmed that lacking Nek7 leads to reduced IL-1β release, lowered recruitment of immune cells, and reduced illness severity in contrast to wild-type mice [179, 180]. Consequently, these results reveal that Nek7 may be a positive regulator of NLRP3 inflammasome activation.

High concentrations of extracellular K^+ inhibit the interplay between Nek7 and NLRP3. Continual efflux is therefore required to maintain this interaction. Decreased intracellular K^+ is prone to generate structural changes in NLRP3 favoring the interaction Nek7/NLRP3. Additionally, it also has been reported that Nek7 is still required for inflammasome activation even if a mutation in NLRP3 that allows K^+ efflux exists. Conclusively, although Nek7 is therefore independently needed for NLRP3 inflammasome activation, a deep comprehension of Nek7 signaling mechanism will contribute to better understanding on how the mechanism of NLRP3-inflammasome activation works [180].

5.3. NLRP3-inflammasome inhibitors

Inflammasome's deregulation is tightly linked to many autoinflammatory and autoimmune diseases, including metabolic disorders, such as type 2 diabetes mellitus, obesity and atherosclerosis as well as neurodegenerative disorders (Alzheimer's disease, Parkinson disease, multiple sclerosis). The current understanding of inflammasome activation and its connection to inflammatory pathologies have therefore, drawn scientific community's attention to develop potential therapies targeting inflammasomes [184].

Based on several preliminary data, the inhibition of NLRP3-inflammasome is maybe one of the most interesting strategies against autoinflammatory and autoimmune diseases. It has been reported that silencing NLRP3 suppresses atherosclerosis and stabilizes plaques in ApoE- deficient mice [185]. The inflammasome inhibitor Arglabin, has shown to reduce atherosclerosis and inflammatory events in ApoE-deficient mice [186]. Coll et al., have recently identified the attractive molecule MCC950, a potent and specific inhibitor of the NLRP3-inflammasome [187]. This molecule was found to halt experimental NLRP3-inflammasome- associated diseases as well as inflammasome ex vivo control in human peripheral blood mononuclear cells (PBMCs) from cryopiryn-associated periodic síndrome (CAPs) patients [187].

Our group has recently investigated the effect of MCC950 on mice fed with different kinds of diet: high-fat diet, high-sugar diet and a combination of both. We observed a very remarkable cardio-protective effect in the absence of NLRP3-inflammasome, either by genetic deletion or by pharmacological inhibition with MCC950 treatment [146].

Hence, a close interplay between inflammation and age-related cardio-metabolic disorders exist. There are too many data showing that NLRP3 has a key role in the onset and development of age-related metabolic disorders, which means that new reseach might be perform in this field to clarify the mechanisms by which the NLRP3-inflammasome is involved in cardio-metabolic events related to aging and how could this affect to life and especially, to healthspan.

In the present Thesis, we will evaluate the involvement of NLRP3-inflammasome in healthspan by studying cardiac and liver function during the aging process.

HYPOTHESIS AND THESIS OUTLINE

We hypothesize that the deletion of NLRP3-inflammasome either genetically, or pharmacologically, might enhance age-related cardio-metabolic complications.

In this Thesis, various approaches are used to evaluate the effect of NLRP3 gene in physiological aging (**overall purpose**).

From this main purpose, arise two other specific aims:

1. To dilucidate the influence of the presence or the absence of NLRP3-inflammasome on the regular biochemistry of the heart as we age (**chapter 02**).

2. To investigate the pharmacological inhibition of NLRP3-inflammasome by the specific molecule MCC950 (**chapter 03**).

Finally, **chapter 04** discusses the most important findings of this Thesis and puts them into a broader outlook of the curret status of the field and the future perspectives.

REFERENCES

1. Kirkwood TB. Understanding the Odd Science of Aging. *Cell.* **2005**, 120: 437-447.

2. Ljubuncic P, Reznick AZ. The evolutionary theories of aging revisited. A mini-review. *Gerontology*. **2009**, 55: 205-216.

3. Jin K. Modern biological theories of aging. *Aging Dis.* **2010**, 1: 72-74.

4. Lipsky MS, King M. Biological Theories of Aging. *Dis Mon.* **2015**, 61: 460-466.

5. Leon LJ, Gustafsson AB. Staying young at heart: autophagy and adaptation to cardiac aging. *J Mol Cell Cardiol.* **2016**, 95: 78-85.

6. North BJ, Sinclair DA. The intersection between aging and cardiovascular disease. *Circ Res.* **2012**, 110: 1097-1108.

7. World Health Organization. Media centre. Mental Health of older adults. Key facts. **2017** (http://www.who.int/mediacentre/factsheets/fs381/en/).

8. Strowig T, Henao-Mejia J, Elinav E, *et al*. Inflammasomes in health and disease. *Nature.* **2012**, 481: 278-286.

9. Hoeijmakers JH. DNA damage, aging, and cancer. *N Engl J Med*. **2009**, 361: 1475-1485.

10. Wu H, Roks AJ. Genomic instability and vascular aging: a focus on nucleotide excsion repair. *Trends Cardiovasc Med*. **2014**, 24: 61-68.

11. Vijg J, Suh Y. Genome stability and aging. *Annu Rev Physiol*. **2013**, 75: 645-668.

12. Hallgren J, Pietrzak M, Rempala G, *et al*. Neurodegeneration-associated instability of ribosomal DNA. *Biochim Biophys Acta*. **2014**, 1842: 860-868.

13. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res*. **1961**, 25: 585-621.

14. Hayflick L. The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res*. **1965**, 37: 614-636.

15. Campisi J. Cancer, aging and cellular senescence. *Vivo (Brooklyn).* **2000**, 14: 183-188.

16. Campisi J, Kim SH, Lim CS, *et al*. Cellular senescence, cancer and aging: the telomere connection. *Exp Gerontol*. **2001**, 36: 1619-1637.

17. Wright WE, Shay JW. Cellular senescence as a tumor protector mechanism. The essential role of counting. *Curr Opin Genet Dev.* **2001**, 11: 98-103.

18. Dimri GP. What has senescence got to do with cancer?. *Cancer cell.* **2005**, 7: 505-512.

19. Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. *Cell.* **2007**, 130: 223-233.

20. Young ARJ, Narita M. SASP reflects senescence. *EMBO Rep*. **2009**, 10: 228-230.

21. Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell*. **2005**, 120: 513-522.

22. Krtolica A, Parrinello S, Lockett PY, *et al.* Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci USA.* **2001**, 98: 12072-12077.

23. McCarthy Da, Clark RR, Bartling TR, *et al*. Redox-control of the senescence regulator Interleukin-1 α and the secretory phenotype. *J Biol Chem*. **2013**, 288. 32149-32159.

24. Xue W, Zender L, Miething C, *et al*. Senescence and tumor clearance is triggered by p53 restoration in murine liver carcinomas. *Nature*. **2007**, 445: 656-660.

25. Krizhanovsky M, Yon RA, Dickins S, *et al*. Senescence of activated stellate cells limits liver fibrosis. *Cell*. **2008**, 134: 657-667.

26. Kuilman T, Michaloglou C, Vredeveld LCWW, *et al*. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell*. **2008**, 133: 1019-1031.

27. Wajapeyee N, Serra RW, Zhu X, *et al*. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by secreted protein IGFBP7. *Cell*. **2008**, 132: 363-374.

28. Baker DJ, Wijshake T, Tchkonia NK, *et al*. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nat Nat Publ Group a Div Macmillan Publ Ltd. All Rights Reserve*. **2011**, 479: 232-236.

29. Byun H, Lee Y, Kim J. From cell senescence to age-related diseases: differential mechanisms of action of senescence-associated secretory phenotypes. *BMB Rep*. **2015**, 48: 549-558.

30. Palmer AK, Tchkonia NK, LeBrasseur EN, *et al*. Cellular senescence in Type 2 Diabetes: a therapeutic opportunity. *Diabetes.* **2015**, 64: 2289-2298.

31. Kunlin J. Modern biological theories of aging. *Aging and Dis.* **2010**, 1:72-74.

32. Peng C, Wang X, Chen J, *et al*. Biology of ageing and role of dietary antioxidants. *Biomed Res Int*. **2014**, 831841.

33. Medawar PB. An unsolved problem of biology. *Evol Heath Dis.* **1952**, 24.

34. Williams GC. Pleiotropy, natural selection, and the evolution of senescence. *Evolution*. **1957**, 11: 398-411.

35. Kirkwood TBL, Austad SN. Why do we age? *Nature.* **2000**, 408: 233-238.

36. Partridge L, Germs D. Mechanisms of aging: public or private? *Nat Rev Genet*. **2002**, 3:165-175.

37. Promislow DE. DNA repair and the evolution of longevity: a critical analysis. *J Theor Biol*. **1994**, 170: 291-300.

38. Bürckle A. Physiology and pathophysiology of poly(ADP-ribosyl)ation. *Bioessays.* **2001**, 23: 795-806.

39. Grube K, Bürckle A. Poly (ADP-ribose) polymerase activity in mononuclear leukocytes of 13 mammalian species correlates with

species-specific life span. *Proc Natl Acad Sci USA.* **1992**, 89: 11759-11763.

40. Muiras M, Müller M, Schächter, *et al*. Increased poly(ADP-ribose) polymerase activity in lymphoblastoid cell lines from centenarians. *J Mol Med*. **1998**, 76: 346-354.

41. Wallace DC. Mitochondrial diseases in man and mouse. *Science.* **1999**, 283: 1482-1488.

42. Müller-Höcker J. Cytochrome-c-oxydase deficient cardiomyocytes in human heart- an age-related phenomenon. A histolochemical ultracytochemical study. *Am J Pathol.* **1989**, 134: 1167-1173.

43. Brierley EJ, Johnson MA, Lightowlers RN, *et al*. Role of mitochondrial DNA mutations in human aging: implications for the central nervous system and muscle. *Ann Neurol.* **1998**, 43: 217-223.

44. Cottrell DA, Ince PG, Blakely EL, *et al.* Neuropathological and histochemical changes in a multiple mitochondrial DNA delection disorder. *J Neuropathol Exp Neurol.* **2000a**, 59: 621-627.

45. Cottrell DA, Blakely EL, Johnson MA, *et al*. Cytochrome c oxidase deficient cells accumulate in the hippocampus and choroid plexus with age. *Neurobiol Aging.* **2000b**, 22: 265-272.

46. Taylor RW, Barron MJ, Borthwick GM, *et al*. Mitochondrial DNA mutations in human colonic crypt stem cells. *J Clin Invest.* **2003**, 112: 1351-1360.

47. Chandrasekaran A, Idelchik MD, Melendez JA. Redox control of senescence and age-related disease. *Redox Biol*. **2017**, 11: 91-102.

48. Kim S, Kaminker P, Campisi J. Telomeres, aging and cancer: in search of a happy ending. *Oncogene*. **2002**, 21: 503-511.

49. Campisi J. Aging, tumor suppression and cancer. High wire-act! *Mech Ageing Dev*. **2005**, 126: 51-58.

50. López-Otin C, Blasco MA. The hallmarks of aging. *Cell*. **2013**, 153: 1194-1217.

51. Soti C, Csermely P. Aging and molecular chaperones. *Exp Gerontol*. **2003**, 38: 1037-1040.

52. Carrard G, Bulteau AL, Petropoulos I, *et al*. Impairment of proteasome structure and function in aging. *Int J Biochem Cell Biol*. **2002**, 34: 1461-1474.

53. Terman A, Brunk UT. Aging as a catabolic malfunction. *Int J Biochem Cell Biol*. **2004**, 36: 2365-2375.

54. Kirkwood TB, Boys RJ, Gillespie CS, *et al*. Towards an e-biology of aging: integrating theory and data. *Nat Rev Mol Cell Biol*. **2003**, 4: 243-249.

55. Lakkata EG, Levy D. Arterial and cardiac aging: Major shareholders in cardiovascular disease enterprises, part I: aging arteries: a "set up" for vascular disease. *Circulation*. **2003**, 107: 139-146.

56. World Health Organization. Programme. Cardiovascular disease. **2016** (http://www.who.int/cardiovascular_diseases/en/).

57. McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the life-span and upon the ultimate body size. *J Nutr*. **1935**, 10-63.

58. Fontana L, Partidge L, Longo VD. Extending healthy life span- from yeast to humans. *Science*. **2010**, 328: 321-326.

59. Catello L, Froio T, Cavallini G, *et al*. Caloric restriction protects against age-related rat aorta aterosclerosis. *FASEB J*. **2005**, 19: 1863-1865.

60. Colman RJ, Anderson RM, Johnson SC, *et al*. Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science*. **2009**, 325: 201-204.

61. Guo Z, Mitchell-Raymundo F, Yang H, *et al*. Dietary restriction reduces atheroscerosis and oxidative stress in the aorta of apolipoprotein E-deficient mice. *Mech Ageing Dev*. **2002**, 123: 1121-1131.

62. Seymur EM, Parikh RV, Singer AA, *et al*. Moderate calorie restriction improves cardiac remodeling and diastolic dysfunction in the Dahl-SS rat. *J Mol Cell Cardiol*. **2006**, 41: 661-668.

63. Taffet GE, Pham TT, Hartley CJ. The age-associated alterations in late diastolic function in mice are improved by caloric restriction. *J Gerontol A Biol Sci Med Sci*. **1997**, 52: B285-B290.

64. Dolinsky VW, Morton JS, Oka T, *et al*. Caloric restriction prevents hypertension and cardiac hypertrophy in the spontaneously hypertensive rat. *Hypertension*. **2010**, 56: 412-421.

65. Liao CY, Rikke BA, Johnson TE, *et al*. Genetic variation in the murine lifespan response to dietary restriction: from life extensión to life shortening. *Aging Cell*. **2010**, 9: 92-95.

66. Swindell WR. Dietary restriction in rats and mice: a meta-analysis and review of the evidence for genotype-dependent effects on lifespan. *Aging Res Rev*. **2012**, 11: 254-270.

67. Harper JM, Leathers CW, Austad SN. Does caloric restriction extend life in wild mice? *Aging Cell*. **2006**, 5: 441-449.

68. Mitchell SJ, Madrigal-Matute J, Scheibye-Knudsen M, *et al*. Effects of sex, strain, and energy intake on hallmarks of aging in mice. *Cell Metabolism*. **2016**, 23: 1093-1112.

69. Fontana L, Meyer TE, Klein S, *et al*. Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. *Proc Nat Acad Sci*. **2004**, 101: 6659-6663.

70. Baur JA, Pearson KJ, Price NL, *et al*. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature*. **2006**, 444: 337-342.

71. Harrison DE, Strong R, Sharp ZD, *et al.* Rapamycin fed late in life extends lifespan in genetically heterogeneus mice. *Nature*. **2009**, 460: 392-395.

72. Alcocer- Gómez E, Garrido- Maraver J, Bullón P, *et al.* Metformin and caloric restriction induce an AMPK-dependent restoration of mitocondrial dysfunction in fibroblasts from Fibromyalgia patients. *Biochim Biophys Acta*. **2015**, 1852: 1257-1267.

73. Martin- Montalvo A, Mercken EM, Mitchell SJ, *et al*. Metformin improves healthspan and lifespan in mice. *Nat Commun*. **2013**, 4: 2192.

74. Marín- Aguilar F, Pavillard LE, Giampieri F, *et al*. Adenosine Monophosphate (AMP)- activated Protein Kinase: a new target for nutraceutical compounds. *Int J Mol Sci*. **2016**, 18: 288.

75. Finkel T. The metabolic regulation of aging. *Nat Med*. **2015**, 21: 1416-1423.

76. Hardie DG. Amp- Activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes Dev.* **2011**, 25: 1895-1908.

77. Kim J, Yoon MY, Choi SL, *et al*. Effects of stimulation of amp-actvated protein kinase on insulin- like growth factor 1- and epidermal growth factor- dependent extracelullar signal- regulated kinase pathway. *J Biol Chem*. **2001**, 276: 19102- 19110.

78. Canto C, Gerhart- Hines Z, Feige JN, *et al*. AMPK regulates energy expenditure by modulating NAD+ metabolism and sirt1 activity. *Nature*. **2009**, 458: 1056- 1060.

79. Fulco M, Sartorelli V. Comparing and contrasting the roles of Ampk and sirt1 in metabolic tissues. *Cell Cycle*. **2008**, 7: 3669- 3679.

80. Russell RR III, Li J, Coven DL, *et al.* Amp-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis and injury. *J Clin Invest*. **2004**, 114: 495- 503.

81. Fu YN, Xiao H, Ma XW, *et al*. Metformin attenuates pressure overload- induced cardiac hypertrophy via ampk activation. *Acta Pharmacol Sin*. **2011**, 32: 879- 887.

82. Blair E, Redwood C, Ashrafian H, *et al*. Mutations in the gamma(2) subunit of amp- activated protein kinase cause familial hypertrophic

cardiomyopathy: evidence for the central role of energy compromise in disease pathogenesis. *Hum Mol Genet*. **2001**, 10: 1215- 1220.

83. Kenyon C, Chang J, Gensch E, *et al*. A C elegans mutant that lives twice as long as wild type. *Nature*. **1993**, 366: 461- 464.

84. Guttridge DC. Signaling pathways weigh in on decisions to make or brake skeletal muscle. *Curr Opin Clin Nutr Metab Care*. **2004**, 7: 443- 450.

85. Li Q, Li B, Wang X, *et al*. Overexpression of insulin-like growth factor-1 in mice protects from myocyte death after infarction, attenuating ventricular dilation, wall stress, and cardiac hypertrophy. *J Clin Invest*. **1997**, 100: 1991- 1999.

86. Kajsura J, Fiordaliso F, Andreoli AM, *et al*. Igf-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II- mediated oxidative stress. *Diabetes*. **2001**, 50: 1414- 1424.

87. Delaughter MC, Taffet GE, FiorottoML, *et al*. Local insulin- like growth factor I expression induces physiologic, then pathologic, cardiac hypertrophy in trangenic mice. *FASEB J*. **1999**, 13: 1923- 1929.

88. Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol*. **2010**, 5: 253- 295.

89. Kaeberlein M, McVey M, Guarente L. The sir2/3/4 complex and sir2 alone promote longevity in saccharomyces cerevisiae by two different mechanisms. *Genes Dev*. **1999**, 13: 2570- 2580.

90. Mostoslavsky R, Chua KF, Lombard DB, *et al*. Genomic instability and aging- like phenotype in the absence of mammalian sirt6. *Cell*. **2006**, 124: 315- 329.

91. Rogina B, Helfand SL. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci USA*. **2004**, 101: 15998- 16003.

92. Tissenbaum HA, Guarente L. Increased dosage of a sir2- gene extends lifespan in Caenorhabditis elegans. *Nature*. **2001**, 410: 227-230.

93. Vakhrusheva O, Smolka C, Gajawada P, *et al*. Sirt7 increases stress resistance of cardiomyocytes and prevents apoptosis and inflammatory cardiomyopathy in mice. *Circ Res*. **2008**, 102: 703-710.

94. Lan F, Cacicedo JM, Ruderman N, *et al*. Sirt1 modulation of the acetylation status, cytosolic localization, and activity of LKB1: possible role in amp-activated protein kinase activation. *J Biol Chem*. **2008**, 283: 27628-27635.

95. Bordone L, Motta MC, Picard F, *et al*. Sirt1 regulates insulin secretion by repressing ucp2 in pancreatic beta cells. *PLoS Biol*. **2006**, 4: e31.

96. Ghosh HS, McBurney M, Robbins PD. Sirt1 negatively regulates the mammalian target of rapamycin. *PLoS One.* **2010**, 5: e9199.

97. Liu M, Wilk SA, Wang A, *et al*. Resveratrol inhibits mTOR signaling by promoting the interaction between mTOR and depTOR. *J Biol Chem*. **2010**, 285: 36387-36394.

98. Hsu ZP, Zhai P, Yamamoto T, *et al*. Silent information regulator 1 protects the heart from ischemia/ reperfusion. *Circulation*. **2010**, 122: 2170-2182.

99. Kawashima T, Inuzuka Y, Okuda J, *et al*. Constitutive sirt1 overexpression impairs mitochondria and reduces cardiac function in mice. *J Molec Cell Cardiol*. **2011**, 51: 1026-1036.

100. Alcendor RR, Gao S, Zhai P, *et al*. Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res*. **2007**, 100: 1512-1521.

101. Hafner AV, Dai J, Gomes AP, *et al*. Regulation of the mptp by the sirt3-mediated deacetylation of cypd at lysine 166 suppresses age-related cardiac hypertrophy. *Aging (Albany NY)*. **2010**, 2: 914-923.

102. Sundaresan NR, Gupta M, Kim G, *et al*. Sirt3 blocks the cardiac hypertrophic response by augmenting foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest*. **2009**, 119: 2758-2771.

103. Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. *Nat Rev Cancer*. **2004**, 4: 335-348.

104. Li G, Kim SG, Blenis J. Rapamycin: one drug, many effects. *Cell Metab*. **2014**, 19: 373-379.

105. Kaeberlein M, Powers RW III, Steffen KK, *et al*. Regulation of yeast replicative life span by TOR and sch9 in response to nutrients. *Science*. **2005**, 310: 1193-1196.

106. Kapahi P, Zid BM, Harper T, *et al*. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr Biol*. **2004**, 14: 885-890.

107. Vellai T, Takacs- Vellai K, Zhang Y, *et al*. Genetics: influence of TOR kinase on lifespan in C Elegans. *Nature*. **2003**, 426: 620.

108. McMullen JR, Sherwood MC, Tarnavsky O, *et al*. Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. *Circulation*. **2004**, 109: 3050-3055.

109. Shioi T, McMullen JR, Tarnavsky O, *et al*. Rapamycin attenuates load- induced cardiac hypertrophy in mice. *Circulation*. **2003**, 107: 1664-1670.

110. Halapas A, Armakolas A, Koutsilieris M. Autophagy: a target for myocardial interventions in myocardial pathophysiology. *Exp Opin Ther Targets*. **2008**, 12: 1509-1522.

111. Yamaguchi O, Otsu K. Role of autophagy in aging. *Journal of cardiovascular pharmacology*. **2012**, 60: 242-247.

112. Uddin MN, Nishio N, Ito S, *et al*. Autophagic activity in thymus and liver during aging. *Age (Dordr)*. **2012**, 34 :75-85.

113. Rubinsztein DC, Marino G, Kroemer G. Autophagy and aging. *Cell*. **2011**, 146: 682- 695.

114. Cuervo AM. Autophagy and aging: keeping that old broom working. *Trends Genet*. **2008**, 24: 604- 612.

115. Inoki K, Li Y, Zhu T, *et al*. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nature Cell Biol*. **2002**, 4: 648-657.

116. Manning BD, Tee AR, Logsdon MN, *et al*. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Molecular Cell*. **2002**, 10: 151-162.

117. Potter CJ, Pedraza LG, Xu T. Akt regulates growth by directly phosphorylating Tsc2. *Nature Cell Biol*. **2002**, 4: 658-665.

118. Gwinn DM, Shackelford DB, Egan DF, *et al*. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Moll Cell*. **2008**, 30: 214-226.

119. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell*. **2003**, 115: 577-590.

120. Kim J, Kundu M, Viollet B, *et al*. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol*. **2011**, 13: 132-141.

121. Kim J, Kim YC, Fang C, *et al*. Differential regultion of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. *Cell*. **2013**, 152: 290-303.

122. Kang R, Zeh HJ, Lotze MT, *et al*. The Beclin1 network regulates autophagy and apoptosis. *Cell Death Differ*. **2011**, 18: 571-580.

123. Mizushima N, Yoshimori T, Oshumi Y. The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol*. **2011**, 27: 107-132.

124. Pankiv S, Clausen TH, Lamark T, *et al*. P62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem*. **2007**, 282: 24131-24145.

125. Leon LJ, Gustafsson AB. Staying young at heart: Autophagy and adaptation to cardiac aging. *J Mol Cell Cardiol*. **2016**, 95: 78-85.

126. Geisler S, Holmstrom KM, Skujat D, *et al*. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol*. **2010**, 12: 119-131.

127. Narendra D, Tanaka A, Suen DF, *et al.* Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol*. ***2008***, 183: 795-803.

128. Kwon J, Han E, Bui CB, *et al*. Assurance of mitochondrial integrity and mammalian longevity by the p62-Keapl-Nrf2-Nqol cascade. *EMBO Rep*. **2012**, 13: 150-156.

129. Simonsen A, Cumming RC, Brech A, *et al*. Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. *Autophagy*. **2008**, 4: 176-184.

130. Morselli E, Maiuri MC, Markaki M, *et al*. Caloric restriction and resveratrol promote longevity through the Sirtuin-1 dependent induction of autophagy. *Cell Death Dis*. **2010**, 1: e10.

131. Han X, Turdi S, Hu N, *et al*. Influence of long-term caloric restriction on myocardial and cardiomyocyte contractile function and autophagy in mice. *The Journal of nutritional biochemistry*. **2012**, 23: 1592-1599.

132. Eisenberg T, Knauer H, Schauer A, *et al*. Induction of autophagy by spermidine promotes longeity. *Nat Cell Biol*. **2009**, 11: 1305-1314.

133. Pyo JO, Yoo SM, Ahn HH, *et al*. Overexpression of Atg5 in mice activates autophagy and extends lifespan. *Nat Commun*. **2013**, 4: 2300.

134. Taneike M, Yamaguchi O, Nakai A, *et al*. Inhibition of autophagy in the heart induces age-related cardiomyopathy. *Autophagy*. **2010**, 6: 600- 606.

135. Bjedov I, Toivonen JM, Kerr F, *et al*. Mechanisms of life span extensión by rapamycin in the fruit fly Drosophila melanogaster. *Cell Metab*. **2010**, 11: 35-46.

136. Anisimov VN, Zabezhinski MA, Popovich IG, *et al*. Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. *Cell Cycle*. **2011**, 10: 4230-4236.

137. Wu JJ, Liu J, Chen EB, *et al*. Increased mammalian lifespan and a segmental tissue-specific slowing of aging after genetic reduction of mTOR expression. *Cell Rep*. **2013**, 4: 913-920.

138. Hsu HC, Chen CY, Lee BC, *et al*. High-fat diet induces cardiomyocyte apoptosis via the inhibition of autophagy. *European journal of nutrition.* **2015.**

139. Wohlgemuth SE, Julian D, Akin DE, *et al*. Autophagy in the heart and liver during normal aging and calorie restriction. *Rejuvenation Res*. **2007**, 10: 281-292.

140. Flynn JM, O'Leary MN, Zambataro CA, *et al*. Late-life rapamycin treatment reverses age-related heart dysfunction. *Aging Cell*. **2013**, 12: 851-862.

141. Boyle AJ, Shih H, Hwng J, *et al*. Cardiomyopathy of aging in the mammalian heart is characterized by myocardial hypertrophy, fibrosis and a predisposition towards cardiomyocyte apoptosis and autophagy. *Exp Gerontol*. **2011**, 46: 549-559.

142. Zhou J, Freeman TA, Ahmad F, *et al*. GSK-3 alpha is a central regulator of age-related pathologies in mice. *J Clin Invest*. **2013**, 46: 549-559.

143. Goldberg EL, Dixit VD. Drivers of age-related inflammation and strategies for health span extension. *Immunol Rev*. **2015**, 265: 63-74.

144. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*. **2002**, 10: 417-426.

145. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol.* **2011**, 29: 707-735.

146. Pavillard LE, Cañadas-Lozano D, Alcocer-Gómez E, *et al*. NLRP3-inflammasome inhibition prevents high fat and high sugar diets-induced heart damage through autophagy induction. *Oncotarget*. **2017**, 8: 99740-99756.

147. Pavillard LE[1], Marín-Aguilar F[1], Bullón P, *et al.* Cardiovascular disease, NLRP3-inflammasome, and western-type dietary patterns. *Pharmacol Res*. **2018**, 131: 44-50.

148. Franceschi C, Bonafè M, Valensin S, *et al*. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci*. **2000**, 908: 244-254.

149. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-related diseases. *J Gerontol A Biol Sci Med Sci.* **2014**, 69: S4-S9.

150. Schroder K, Tschopp J. The inflammasomes. *Cell.* **2010**, 140: 821-832.

151. Ting JP, Lovering RC, Alnemri ES, *et al.* The NLR gene family: a standard nomenclature. *Immunity*. **2008**, 28:285-287.

152. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanisms of action, role in disease, and therapeutics. *Nat Med.* **2015**, 21:677-687.

153. Kayagaki N, Warming S, Lamkanfi M, *et al*. Non-canonical inflammasome activation targets caspase-11. *Nature.* **2011**, 479: 117-121.

154. Lamkanfi M, Kanneganti TD. Nlrp3: an immune sensor of cellular stress and infection. *Int J Biochem Cell Biol.* **2010**, 42: 792–795.

155. Kanneganti TD, Lamkanfi M, Nunez G. Intracellular NOD-like receptors in host defense and disease. *Immunity.* **2007**, 27: 549–559.

156. Tschopp J, Schroder K. NLRP3 inflammasome activation: The convergence of multiple signalling pathways on ROS production? *Nat Rev Immunol.* **2010**, 10: 210–215.

157. Bauernfeind FG, Horvath G, Stutz A, *et al*. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol*. **2009**, 183: 787-791.

158. Barker BR, Taxman DJ, Ting JP. Cross-regulation between the IL-1B/IL-18 processing inflammasome and other inflammatory cytokines. *Curr Open Immunol*. **2011**, 23: 591-597.

159. Strowig T, Henao-Mejia J, Elinav E, *et al*. Inflammasomes in health and disease. *Nature.* **2012**, 481: 278-286.

160. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol.* **2013**, 13: 397-411.

161. de Zoete MR, Palm NW, Zhu S, *et al*. Inflammasomes. *Cold Spring Harb Perspect Biol*. **2014**, 6:a016287.

162. Aachoui Y, Sagulenko V, Miao EA, *et al*. Inflammasome-mediated pyroptotic and apoptotic cell death, and defense against infection. *Curr Opin Microbiol*. **2013**, 16: 319–26.

163. Dostert C, Petrilli V, Van Bruggen R, *et al*. Innate immune activation through Nalp3 inflammasome sensing asbestos and silica. *Science.* **2008**, 320: 674-677.

164. Mariathasan S, Weiss DS, Newton K, *et al* . Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*. **2006**, 440: 228-232.

165. Martinon F, Petrilli V, Mayor A, *et al*. Gout- associated uric acid crystals activate the NALP3 inflammasome. *Nature*. **2006**, 440: 237-241.

166. Goldberg EL, Dixit VD. Drivers of age-related inflammation and strategies for health span extension. *Immunol Rev*. **2015**, 265: 63-74.

167. Wang S, Miura M, Jung YK, *et al*. Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell*. **1998**, 92: 501-509.

168. Case CL, Kohler LJ, Lima JB, *et al*. Caspase-11 stimulates rapid fagellin-independent pyroptosis in response to Legionella pneumophila. *Proc Natl Acad Sci USA*. **2013**, 110: 1851-1856.

169. Broz P, Ruby T, Belhocine K, *et al*. Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. *Nature*. **2012**, 490: 288–291.

170. Gurung P, Malireddi RK, Anand PK, *et al.* Toll or interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-beta (TRIF)-mediated caspase-11 protease production integrates Toll-like receptor 4 (TLR4) protein- and Nlrp3 inflammasome-mediated host defense against enteropathogens. *J Biol Chem*. **2012**. 287: 34474-34483.

171. Rathinam VA, Vanaja SK, Waggoner L, *et al*. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell*. **2012**, 150: 606–619.

172. Schauvliege R, Vanrobaeys J, Schotte P, *et al.* Caspase-11 gene expression in response to lipopolysaccharide and interferon-gamma requires nuclear factor-kappa B and signal transducer and activator of transcription (STAT) 1. *J Biol Chem*. **2002**, 277: 41624-41630.

173. Viganò E, Mortellaro A. Caspase-11: the driving factor for noncanonical inflammasomes. *Eur J Immunol.* **2013**, 43: 2240-2245.

174. Shi J, Zhao Y, Wang Y, *et al.* Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature*. **2014**, 514: 187-192.

175. Kayagaki N, Stowe IB, Lee BL, *et al.* Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature.* **2015**, 526: 666-671.

176. Shi J, Zhao Y, Wang K, *et al.* Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature.* **2015**, 526: 660-5.

177. Shenoy AR, Wellington DA, Kumar P, *et al*. GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. *Science*. **2012**, 336: 481–485.

178. Lu B, Nakamura T, Inouye K, *et al*. Novel role of PKR in inflammasome activation and HMGB1 release. *Nature*. 2012, 488: 670–674.

179. Shi H, Wang Y, Li X, *et al.* NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component. *Nat Immunol*. **2016**, 17: 250–258.

180. He Y, Zeng MY, Yang D, *et al*. NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. *Nature*. **2016**, 530: 354–357.

181. Schmid-Burgk JL, Chauhan D, Schmidt T, *et al.* A Genome-wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an Essential Component of NLRP3 Inflammasome Activation. *J Biol Chem*. **2016**, 291: 103–109.

182. Fry AM, O'Regan L, Sabir SR, *et al*. Cell cycle regulation by the NEK family of protein kinases. *J Cell Sci*. **2012**, 125:4423–4433.

183. Salem H, Rachmin I, Yissachar N, *et al*. Nek7 kinase targeting leads to early mortality, cytokinesis disturbance and polyploidy. *Oncogene*. **2010**, 29: 4046–4057.

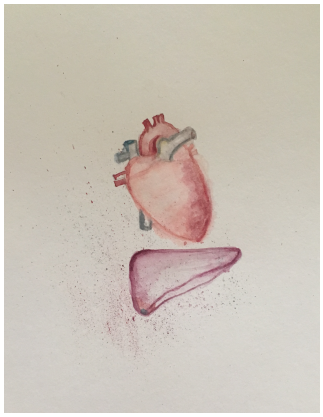
184. Youm YH, Grant RW, McCabe LR, *et al.* Canonical Nlrp3 inflammasome links systemic low-grade inflammation to functional decline in aging. *Cell Metab*. **2013**, 18:519-532.

185. Zheng M, Wei J, Tang Y, *et al*. ApoE-deficient promotes blood-brain barrier disruption in experimental autoinmune encephalomyelitis via alteration of MMP-9. *J Mol Neurosci*. **2014**, 54:282-290.

186. Abderrazak A, Couchie D, Mahmood DF, *et al*. Anti-inflammatory and antiatherogenic effects of the NLRP3 inflammasome inhibitor arglabin in ApoE2.Ki mice fed a high-fat diet. *Circulation*. **2015**, 131: 1061-1070.

187. Coll CR, Robertson AA, Chae JJ *et al*. A small-molecule inhibitor of the NLRP3-inflammasome for the treatment of inflammatory diseases. *Nat Med.* **2015**, 21: 248-255.

CHAPTER 03



Inhibition of the NLRP3-inflammasome by MCC950 improves healthspan in aged mice

Fabiola Marín-Aguilar, Beatriz Castejón-Vega, Elísabet Alcocer-Gómez, Macarena López-Sánchez, Avril A B Robertson, Matthew A Cooper, Eloisa Andújar-Pulido, Mónica Pérez-Alegre, Jordi Muntané, Antonio J Pérez-Pulido, Jesús Ruíz-Cabello, Pedro Bullón, Mario D Cordero

Nature Communications

Under Review

ABSTRACT

The NLRP3-inflammasome has arisen as a key element of inflammatory events that typically occur during aging, being responsible for several age-associated metabolic disorders.

Based on previous data, NLRP3 knockout mice seem to be protected from various aspects of aging decline, but innate immune genetic knockouts may possess aberrant biology. In this study, we investigated the molecular mechanisms by which pharmacological inhibition of NLRP3-inflammasome may improve metabolic changes as we age. NLRP3 inhibition by the selective molecule MCC950 over 12 weeks in C57BL6/J old mice resulted in enhanced metabolic function, mTOR pathway attenuation and subsequent improved healthspan. These findings suggest that the NLRP3-inflammasome inhibitor MCC950 might therefore, be a promising molecule for the treatment of metabolic diseases with a significant impact on healthspan, demonstrating for the first time, the potential of MCC950 molecule as part of an anti-aging approach.

INTRODUCTION

The liver is a human organ able of innate regeneration of lost tissue. Nevertheless, as we age liver functionality and structure, get compromised, what predisposes to metabolic risk. The leading hepatic impairment affecting one-third of the global population is non-alcoholic fatty liver disease (NAFLD) and a common reason of liver transplant [1], easily recognizable by a typical intrahepatic accumulation of lipids. In an environment of oxidative stress and inflammation, NAFLD is prone to evolve into non-alcoholic steatohepatitis (NASH), which constitutes a serious risk factor for the development of cirrhosis and hepatocellular carcinoma **[Figure 1]**.

Recent research based on animal [2, 3] and human data [4] have connected NASH pathogenesis with peroxisome proliferator-activated receptors (PPARs). PPARs belong to subfamily 1 of the nuclear receptor superfamily [5, 6], which consist of 3 different isotypes: α, β/δ and γ. Generally, PPARs form heterodimers with retinoid X receptors, enabling the transcription of diverse target genes. PPARα, in particular, is expressed in metabolically active tissues, and constitutes a major regulator of lipid metabolism in liver [7]. It regulates genes involved in fatty acid β-oxidation, as well as gluconeogenesis and inflammation.

Many data based on animal models and more recently, human data have reported the crucial role of PPARα on the regulation of NASH, visceral adiposity and insulin resistance as well as its positive correlation with adiponectin, suggesting that PPARα might be an interesting therapeutic target for NASH treatment [4].

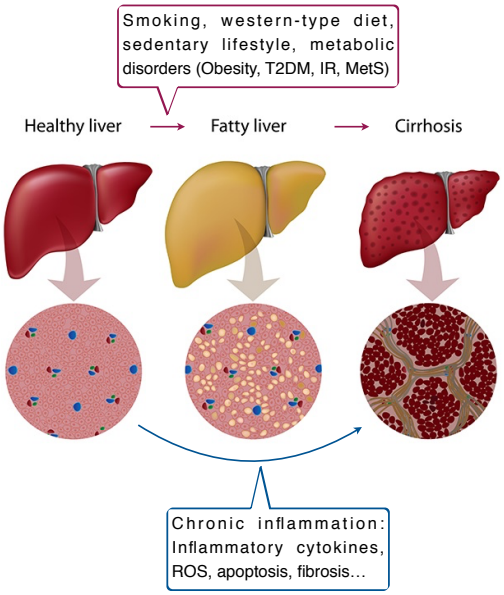


Figure 1. Schematic image representation of different hepatic scenarios. Progression of liver disease as we age and the respective molecular and lifestyle changes influencing hepatic state T2DM, type 2 diabetes dellitus, IR, insulin resistance, MetS, metabolic syndorme. Image credit modified from: https://www.matinasbiopharma.com/business-development/mat8800-for-treatment-of-fatty-liver-disease

On the other hand, chronic inflammation and aging, are also main responsables for liver disability. As first describe Franceschi et al., in 2002 [8], the inflammaging theory arised as a result of a lack of immune system response caused by an imbalance between incremented inflammatory markers and decreased antioxidant enzymes that lies in the characteristic chronic low-grade inflammation developed during the aging process.

As stated in this theory, the persistence of an inflammatory response over years symbolizes the resulting biological susceptibility to age-related diseases [Figure 1]. Preventing, as far as possible, chronic

low-grade inflammation in aged people with a suitable lifestyle, based on regular physical activity and reduced caloric intake, for example, may be essential to promote healthspan.

NLRP3-inflammasome and its association with inflammatory diseases, constitutes a great candidate to target hepatic inflammation and the metabolic events that may trigger during aging. An appealing inhibitor of NLRP3-inflammasome is MCC950.

MCC950 is a small and selective molecule inhibitor of the canonical and non-canonical NLRP3-inflammasome activation [9]. MCC950 blocks IL-1β secretion and ASC oligomerization induced by active NLRP3 in mouse and human macrophages. It also decreases IL-1β and IL-18 release, which mitigates the severity of CAPS in mouse models. Furthermore, what is also very interesting is that MCC950 action is specifically on NLRP3, since it does not inhibit NLRP1, AIM2, NLRC4-inflammasomes activation [9].

Structurally, MCC950 consists of a dyarylsulfonilurea-derived compound **[Figure 2]**. Sulfonylureas are widely used as antidiabetic treatment and their main pharmacological effect is to rise in plasma insulin concentrations. This fact occurs for two reasons: because there is stimulation of insulin secretion by pancreatic β-cells, and in the second place, because there is a decrease in hepatic insulin clearance, what makes MCC950 molecule highly attractive to target age-realted hepatic dysfunctionality.

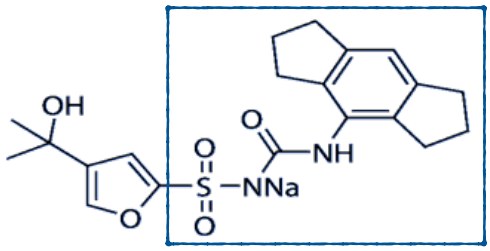


Figure 2. MCC950 molecule. Chemical structure of MCC950 molecule. Coloured box indicates sulfonylurea group.

Some studies have reported the interest of MCC950 on NASH pathology, since it showed improved NAFLD and liver fibrosis in obese diabetic mice when NLRP3-inflammasome was inhibited [10].

Further studies to test MCC950 molecule have been performed. After evaluating the influence of NLRP3 on heart tissue from mice, they concluded that pharmacological inhibition of NLRP3-inflammasome enhanced metabolic and inflammatory events in animal hearts as well as in vitro [11].

MATERIALS AND METHODS

Mice and animal care

Animal studies were performed in accordance with the European Union guidelines (2010/63/EU) and the corresponding Spanish regulations for the use of laboratory animals in chronic experiments (RD 53/2013 on the care of experimental animals). All experiments were approved by the local institutional animal care committee.

For all experiments, male C57/BL6/J mice were used, weighing 30-35 g and maintained on a regular 12 h light/dark cycle. Animals were randomized into two different groups and at 16 weeks of age, MCC950 treatment started. Daily doses of 20 mg/kg (drug/kg body weight) were administred intraperitoneally (i.p). All groups had ad libitum access to regular chow and water throughout the study. Regular chow or 14% protein rodent maintenance diet, (carbohydrate:protein:fat ratio of 48:14:4 percent of kcal), was acquired from Teklad Global Laboratories, Envigo. Body weight and food intake were monitored weekly. Animal rooms were maintained at 20–22°C with 30–70% of relative humidity.

In vitro cell experiments

HepG2 cells, an immortalized cell line consisting of human liver carcinoma cells, were cultured at 37°C in a 5% CO_2 atmosphere in DMEM medium supplemented with an antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO, USA), and 10% fetal bovine serum. HepG2 cells were incubated for 24 h with media containing 0.6, 1.2 and 2.4 μM MCC950 corresponding doses.

Reagents

MCC950 was provided by Robertson AAB and Cooper MA [9], from the Institute for Molecular Bioscience, (University of Queensland, Brisbane, Australia). Cocktail of protease inhibitors for protein homogenization (Complete™ Protease Inhibitor Cocktail) was purchased from Boehringer Mannheim (Indianapolis, IN). Bradford reagent for protein concentration assay was purchased from Bio-Rad (Madrid, Spain).

Oral Glucose Tolerance Test (GTT)

For oral GTT, mice were orally given glucose at 2g/kg bodyweight after overnight fasting. Glucose levels in tail blood were measured using a glucometer (Breeze 2, Bayer Health Care, USA) at 0, 15, 30, 60, and 120 minutes time points.

Insulin Growth Factor 1 (IGF-1)

Serum levels of insulin growth factor 1 (IGF-1) were assayed using commercial ELISA kits (R&D Systems, Minneapolis, USA).

Glucose serum levels

Serum levels of glucose were assayed using commercial kits (Randox Laboratories, Antrim, UK).

Leptin and adiponectin

Serum levels of leptin and adiponectin were assayed using commercial ELISA kits (R&D Systems, Minneapolis, USA).

Histology

After anesthesia of mice, livers were excised and immediately placed in a 10% neutral-buffered formalin at room temperature for 24 hours after a brief rinse with PBS. The specimens were embedded in paraffin, cut in 5-µm sections, and stained with hematoxylin and eosin. Liver cross-sectional areas were calculated on a digital microscope (×400) with ImageJ (version 1.34S) software. Masson's trichrome staining was used to detect fibrosis in liver sections and fibrotic areas were also calculated on a digital microscope (×400) with ImageJ (version 1.34S) software.

Western Blot Analysis

Mice livers were collected and immediately snap frozen and homogenized for western blot analysis. Protein concentration was measured using a protein assay reagent (Bio-Rad, Madrid, Spain) according to the Bradford's method. The immunoblot analysis was performed using 20 μg of total protein, separated on SDS PAGE 12% acrilamide gels (Biorad Laboratories Inc., Hercules, CA, USA) and transferred onto a nitrocellulose 0.2 μm membrane. Membrane was blocked in blottinng-grade blocker buffer (Biorad Laboratories Inc., Hercules, CA, USA) for 2 hours. After washing, the immunoblotting was performed at 4°C overnight with shaking using primary antibodies (1:1000) against Sirt1, AKT, pAKT, Beclin1, LC3, Parkin, Bcl2, BAX, Caspase-3, PPARα, and GAPDH (Santa Cruz Biotechnology, USA), AMPK, p-AMPK, PI3K, p-PI3K, mTOR, p-mTOR, SQSTM1/p62 (Cell Signaling, USA). After rinsing, the membranes were incubated in a 1 : 10000 dilution with the corresponding secondary antibody coupled to horseradish peroxidase-conjugated (HPR) at room temperature for 1 hour. Goat anti-rabbit IgG, goat anti-mouse and rabbit anti-goat were

acquired from Calbiochem-Merck Ltd (Nottingham, UK). To prove equal loading, the blots were analyzed for GAPDH expression using an anti-GAPDH antibody ((Santa Cruz Biotechnology). Immunodetection was performed using WesternSure chemiluminescent light-detecting kit (LI-COR Biosciencies, USA). The immunosignals were capture using C-Digit blot scanner and image studio Digits software (LI-COR Biosciencies, USA). Densitometric data were recorded following normalization to the loading control. The signals were analyzed and quantified by an Image Processing and Analisys System in Java (Image J Software, Softonic).

Microarray Analysis

After twelve week-treatment, the liver from vehicle (22 months, n=3) and MCC950-treated (22 months, n=3) WT male mice were used to extract total RNA (RNeasy, QIAGEN, Valencia, CA). Quality of total RNA for array analysis was ascertained using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Biological replicates were synthetized, amplified, purified and the corresponding ss-DNA was labelled with biotin according to the manufacturer's protocol (Affymetrix). 100 ng of total RNA was hybridized to 5.2 µg of labelled cDNA. Then, hybridation, rinsing and scanning of the signal (Scanner 3000 7G, Affymetrix) were performed. Array data were processed using Affymetrix® Genechip® Command Console® 2.0 with respect to background subtraction and normalization. We used the Affymetrix Clariom^{TM} D assay mouse, which includes 65956 genes. The raw data were analysed by the Affymetrix software Trancriptome Analysis Console. Expression values were normalized by SST (Signal Space Transformation)-RMA (Robust Microarray Analysis; *Irizarry et al. 2003*), and the treatment was compared versus control, using the 3 replicates for every analysis. Then, a fold change and p-value was calculated for

every gene by a unpaired test one-way (single factor) using the NMATH package. A gene was considered as differentially expressed when it had a fold change equal or higher than 1.5, and a p-value equal or lower than 0.05. Then, a functional enrichment was performed with the differentially expressed genes using the functional annotation tool from the DAVID web site (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3381967/). So, we found a number of annotation categories. To analyse the expression profile for the genes included in these categories, a heatmap was created for every annotation category, using the heatmap.2 function in the gplots R package, and considering normalized values by z-scores, and making a clustering based on the expression profiles and the average method. Finally, a mean of the expression values in every annotation category was calculated and it was again plotted in a heatmap.

Statistical Analysis

All values are expressed as arithmetic means ± SEM. Data were evaluated using Prism software version 6.0c (GraphPad, San Diego, CA). Statistical differences among the different groups were measured using either an unpaired Student t test or 1-way analysis of variance (ANOVA) when appropriate with Tukeys post-hoc test. A P value of ≤0.05 was considered statistically significant. Asterisks in the figures represent the following: *: P ≤0.05; **: P ≤ 0.01; and ***: P ≤ 0.001.

RESULTS

Pharmacological inhibition of NLRP3-inflammasome prevents from metabolic disorder development in aged WT mice

To test whether pharmacological inhibition of NLRP3-inflammasome could prevent from metabolic events onset, we performed a short-term assay using daily administration of the specific inhibitor of NLRP3, MCC950 [9]. 16-month-old male mice fed with standard diet (SD) and ad libitum conditions of food and drink access were used for these assays.

Dynamic changes in body weight and food intake were monitored weekly during 12 weeks. Significant progressive decrease in body weight was observed shortly after exposing mice to MCC950-treatment **[Figure 1A]**. Although food consumption seemed to remain unsignificant in both groups **[Figure 1B]**, absolute food intake signifincantly decreased in MCC950-treated group, whereas relative food intake continued unarltered during the entire observation period **[Figure 1C]**.

We also examined the effect of MCC950 on glycemia in overnight fasted WT aged mice where MCC950 seemed to regulate serum glucose levels. A reduced AUC in this group was also observed **[Figure 1D]**. Consistent with this, basal levels of glucose and insulin-like growth factor-1 (IGF-1) in serum were decreased in MCC950-treated animals **[Figure 1E]**.

Additional parameters, such as leptin and adiponectin serum levels were assayed. Adiponectin has lately drawn considerable attention due to its potential anti-diabetic effects. This hormone is able to control glucose and lipid homeostasis within the liver. It has also been reported that circulating adiponectin reduced plasma glucose levels in WT mice [12]. With this in mind, significant lower serum leptin levels and leptin/adiponectin ratio were detected in MCC950-treated old mice compared to old vehicle **[Figure 1F]**, which is consistent with the metabolic parameters abovementioned. Plasma lipid levels were reduced in MCC950-treated mice accompanied by a significant reduction in hepatic transaminases, creatine phosphokinase and lactate dehydrogenase, among other serum biomarkers **[Supplemental table 1].**

In summary, the global beneficial metabolic effects of MCC950 have been attributed to an improvement in serum glucose levels and insulin resistance, as well as a correct balance between leptin and adiponectin adipokines which, somehow contributes to improve healthspan in old mice.

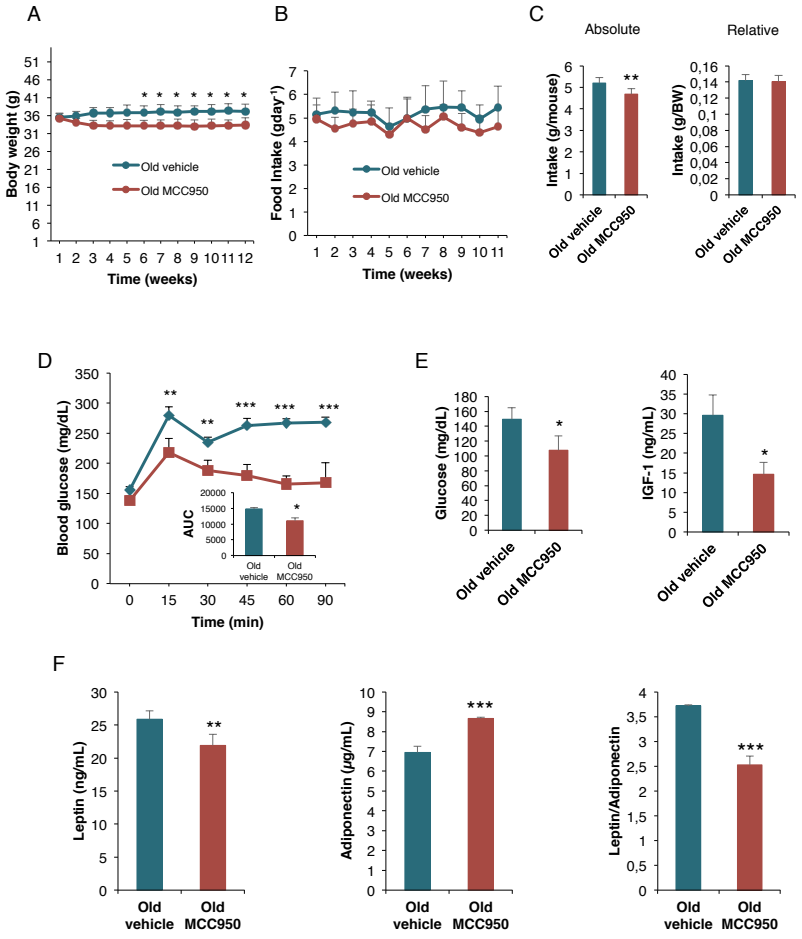


Figure 1. Metabolic effects of NLRP3-inflammasome inhibition in vivo.

(A) Body weight curves during 12 week of MCC950 (20mg/kg) intraperitoneal (i.p) administration. (B) Food intake curves during 12 week-treatment with MCC950 i.p (C) Absolute (left) and relative (right) food intake during treatment of old WT mice. Intake was measured weekly during the entire treatment period. (D) Glucose serum levels at the indicated times after MCC950 20 mg/kg i.p administration by injection and the total area under the curve (AUC) for GTT in WT vehicle and MCC950-treated mice. Mice were orally given glucose at 2 g/kg after overnight fasting. (E) Left panel, glucose serum levels of ad libitum-fed mice. Right panel, insulin-like growth factor-1 serum levels of ad libitum-fed mice. (F) Ad libitum leptin (left), adiponectin (middle), and leptin/adiponectin ratio serum levels (right) at the end of the treatment. All mice were 22 months old (WT males n= 5 per

group) when the experiments were performed. Values are expressed as means ± SEM of independent experiments in triplicate .*P<0.05, **P<0.005, ***P<0.001 MCC950 *vs* vehicle.

MCC950 treatment significantly dampened fat accumulation in liver and liver injury

The pathological alterations of MCC950-treated old WT and vehicle WT mice after 12 weeks of treatment were firstly evaluated by morphologic and histological (H&E and Masson trichrome staining) examination. Short-term MCC950 treatment significantly enhanced liver fat accumulation and moderate liver steatosis **[Figure 2A]**. Further examination with Masson trichrome staining revealed hepatic fibrotic fibers in vehicle old mice that were absent in MCC950- treated mice **[Figure 2B]**. Some observational differences could be detected between the two groups. Tissue colour seemed healthier and less pale in MCC950-treated in comparison to the vehicle group. Moreover, vehicle group showed greater empirical tumour incidence **[Figure 2C]** and increased liver relative weight **[Figure 2D]**. The hepatic lipid content test revealed that MCC950 significantly improved liver fat accumulation and a tendency towards reduced levels of total cholesterol **[Figure 2E]** and triglyceride **[Figures 2F]** in liver tissue.

In summary, short-term MCC950 treatment may result in a progressive loss of adiposity during aging and could presumably act as an antitumor agent.

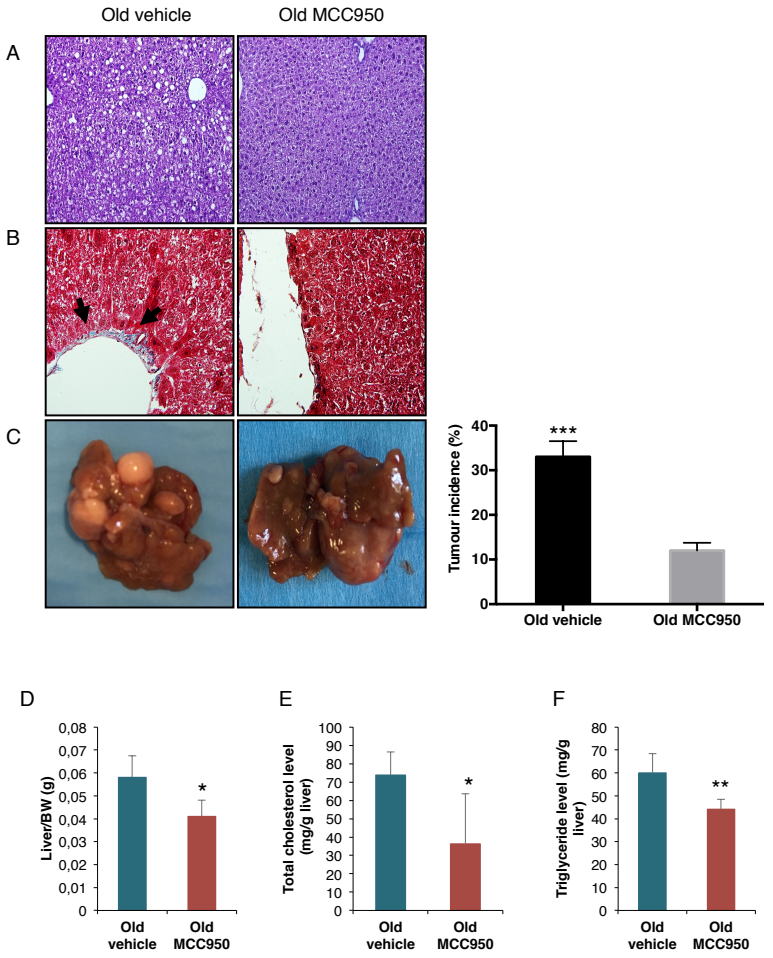


Figure 2. Effect of pharmacological inhibition of NLRP3-inflammasome on liver fat accumulation, hepatic cholesterol and triglycerides. The representative images are represented as follow: (A) H&E staining micrographs of the liver section (400x) after 12-week treatment period, (B) Masson trichrome micrograph of the liver section (200x) after 12-week treatment period. Black arrows show collagen fibrotic fibers, (C) liver morphological photographs after 12-week treatment period and observational tumour incidence, (D) Relative weight of the liver normalized to body weight of ad libitum-fed mice, (E) Ad libitum liver cholesterol levels at the end of the treatment and, (F) ad libitum liver triglyceride content at the end of the treatment. Values are expressed as means ± SEM of independent experiments in triplicate (n= 5 mice for all groups). **P*<0.05, ***P*<0.005, ****P*<0.001 MCC950 *vs* vehicle.

MCC950 treatment may improve longevity network regulation in liver

SIRT-1, AMPK, and TOR are known to be involved in longevity network and lifespan. Sirt-1 is implicated in stress resistance, mitochondrial function, autophagy, and other actions that promote longevity. It has been recently reported that hepatic overexpression of Sirt-1 ameliorates insulin resistance in a mouse model [13], however the exact mechanism by which Sirt-1 cope with certain metabolic processes as we age remain uncertain and we observed no significant changes in protein expression levels **[Figure 3]**. AMPK modulates some important pathways by controlling IGF-1 and constitutes a master governor of metabolism, stress resistance, mitochondrial function and autophagy. Moreover, when AMPK is phosphorylated in a situation of nutrient limiting conditions, mTOR becomes inhibited and viceversa. It has been reported in several studies that low levels of mTOR are linked to lifespan and healthspan both in invertebrates and mammals [14-16]. Consistent with this, upregulators of mTOR pathway, PI3K and AKT, showed significant lower expression levels when active in liver tissue under MCC950 treatment **[Figure 3]**, promoting better liver autophagy as well as controlled protein synthesis and stress resistance. In vitro experiments were performed in Thp1 cells, were NLRP3 and mTOR seemed to modulate each other **[Supplemental figure S1]**.

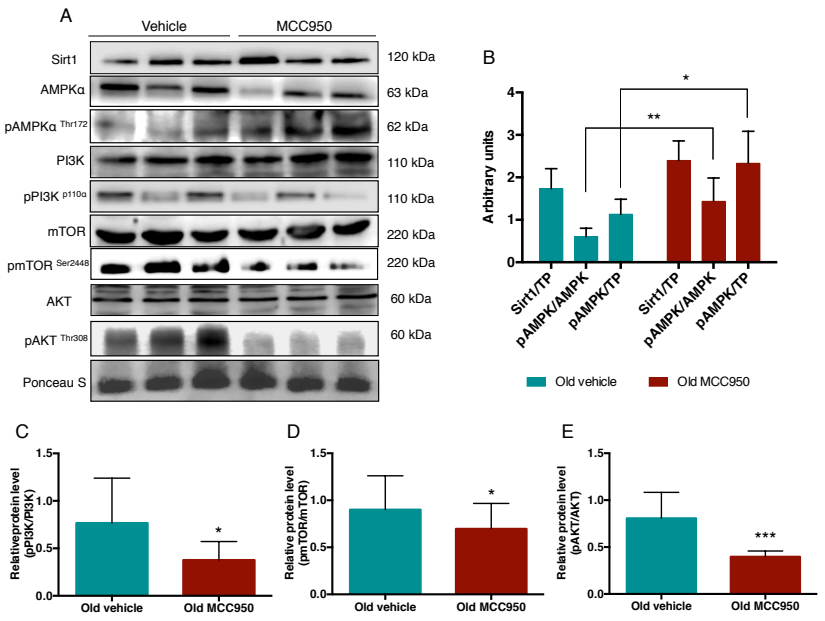


Figure 3. MCC950 controls genes involved in longevity network. Following NLRP3 pharmacological inhibition with MCC950 (20mg/Kg d^{-1} i.p) for 12 weeks, the expression levels of mTOR and p-mTOR were analyzed by Western blotting. The corresponding expression levels of upstream effectors, AMPK, p-AMPK, AKT, p-AKT, PI3K and p-PI3K were also detected, as well as Sirt1. (A) Representative Western blot images of Sirt1, AMPK, p-AMPK, PI3K, p-PI3K, mTOR, p-mTOR, AKT, p-AKT and Ponceau S (loading control), (B) Densitometric quantification of protein expression levels of Sirt1, AMPK and p-AMPK normalized to Ponceau S and represented as relative expression levels, (C-E) Densitometric quantification of protein expression levels of p-PI3K, p-mTOR, p-AKT normalized to Ponceau S and represented as relative expression levels. Values are expressed as means ± SEM of independent experiments in triplicate (n= 5 mice for all groups). **P*<0.05, ***P*<0.005, *** *P*<0.001 MCC950 *vs* vehicle. TP: total protein.

NLRP3-inflammasome inhibition enhances autophagic machinery

The effects of MCC950 on the autophagic flux were investigated in 20-month old WT mice after 12 weeks of treatment. We apparently observed a better autophagic flux in MCC950-treated group, determined by the expression of LC3 II and significant decreased expression of proteins involved in clearance pathways such as SQSTM1/p62 **[Figures 4A and 4B]**. MCC950 also induced higher expression levels of important autophagic proteins, such as Beclin-1 and lower expression of the marker protein parkin, compared with the counterpart vehicle group, although no significant changes were detected **[Figures 4A and 4B]**. Taking into account that LC3 II overexpression may have a dual meaning, since it can evoke either flux autophagy impairment or incremented autophagy, we should interpret LC3 II changes together with p62 results to say that MCC950 may contribute to a more efficient autophagic machinery. However, no significant changes were detected in apoptotic proteins **[Figures 4C and 4D]**.

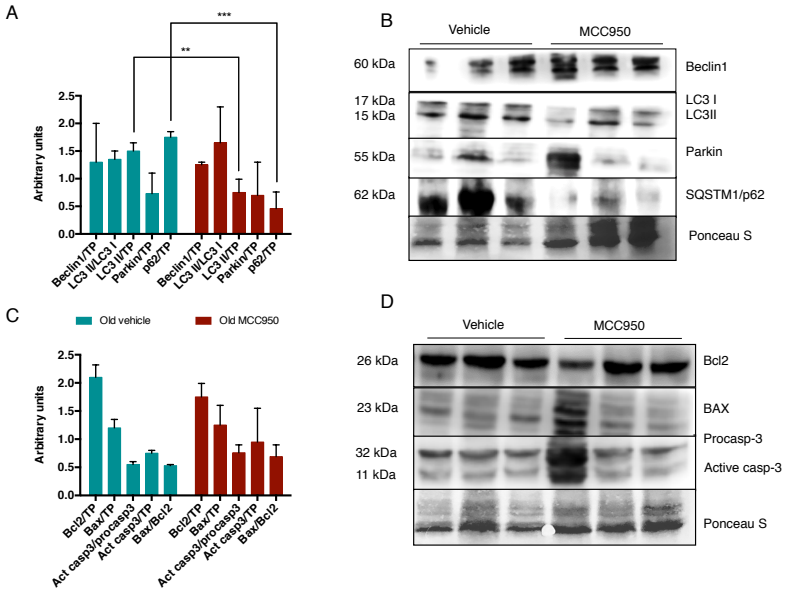


Figure 4. Effect of MCC950 treatment on autophagy signaling and apoptotic pathways. Representative Western blots and densitometric quantifications of (A) SQSTM1/p62, Parkin, LC3 II, LC3 I, Beclin1, (B) Representative Western blot images of Beclin1, LC3 I, LC3 II, Parkin, p62 and Ponceau S (loading control), (C) Densitometric quantifications of active caspase 3, BAX, Bcl2, (D) Representative Western blot images of Bcl2, BAX, procaspase-3, active caspase-3 and Ponceau S (loading control) in response to 12 week MCC950 i.p treatment. Equal loading of proteins is illustrated by Ponceau S bands. Values are expressed as means ± SEM of independent experiments in triplicate (n= 5 mice for all groups). ** P<0.005, *** P<0.001 MCC950 *vs* vehicle.

Pharmacological inhibition of NLRP3-inflammasome protects against age-dependent metabolic disorders in the liver transcriptome

To provide a complete overview, we performed gene expression profiling to further understand NLRP3-inflammasome in liver tissue from old mice. By comparing both groups, we observed some notable differences:

Despite the fact it did not reach the stablished cutt-off for the list of genes involved in lipid metabolism during aging (1.5-fold change) **[Figure 5A]**, PPAR family genes were really close to that limit (1.4-fold change) **[Figure 5B]** and particularly, PPARα resulted very attractive to us because of its involvement in fatty acid ß-oxidation and lipid metabolism in general. In adddition, it has been demonstrated that PPARα protects from steatosis and liver injury in both mice and humans [2-4].

Interestingly, hepatic PPARα was upregulated in MCC950-treated group, which was in concordance with our previous data, showing less lipid accummulation in mice treated with the inhibitor. We decided therefore to study PPARα preotein expression level in vivo and in vitro. Our data provide evidence that under MCC950 treatment, PPARα is upregulated in liver tissue as well as in HepG2, resulting 1.2 μM, the optimal dose in vitro **[Figures 5C and 5D]**. Additional heat maps and de list of all the significantly modified gene sets can be found in **[Supplemental figures S2 and S3 and supplemental tables 2 and 3]**. A subset of expression genes was verified by polymerase chain reaction (qRT-PCR) **[Supplemental figure S4 and supplemental table 4]**.

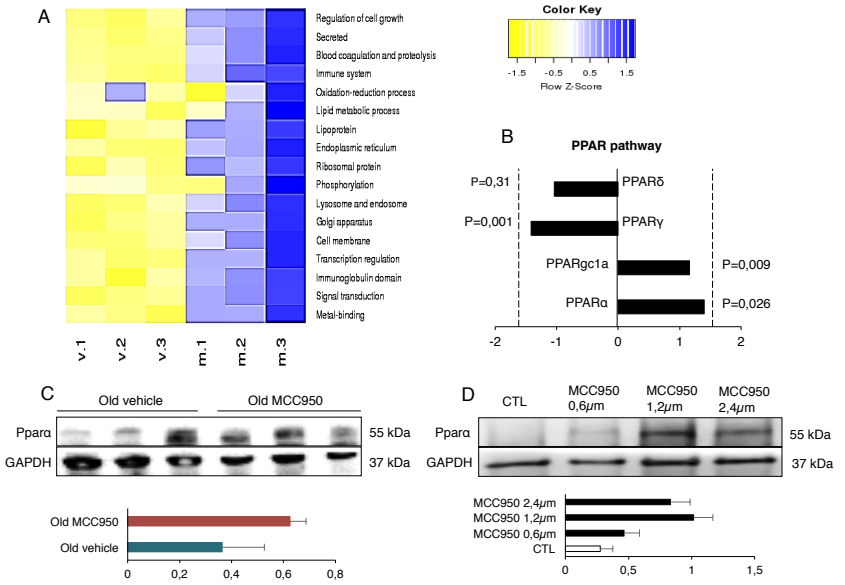


Figure 5. NLRP3-inflammasome regulates age-related changes in liver transcriptome. (A) Gene expression profile comparing genes significantly up-(blue) or down-regulated (yellow) by either vehicle or MCC950-treated mice (Z-ratio), (B) Representative plot of down-(left) or up-regulated (right) PPAR family genes under MCC950 treatment, (C) Representative Western blot images of PPARα and GAPDH (loading control) of mice liver tissue, and the corresponding densitometric quantifications, (D) Representative Western blot images of PPARα and GAPDH (loading control) in vitro, and the corresponding densitometric quantifications. In vitro assay was performed in HepG2 cells and three different doses of MCC950 were assayed. (n= 3 mice per treatment).

DISCUSSION

It is widely known that as we age, we are more prone to develop metabolic events, including increased cholesterol levels, glucose intolerance, insulin resistance, and certain hormone imbalance [17]. All these complications may lead to develop great tendency to metabolic disabilities that may trigger tissue degeneration. Additionally, the typical age-related systemic and chronic inflammatory state improves the chances of insulin resistance onset in muscle and adipose tissue, driving to energy homeostasis imbalance and disturbed cellular functions.

In concordance with the inflammaging theory, it has been reported that reduction of NLRP3-inflammasome dependent proinflammatory cascade, mitigated age-associated deteriorating changes in most organs [18].

In this scenario, research on NLRP3-inflammasome inhibitors has become very attractive. Our findings provide data of how the specific inhibitor MCC950 may be a key component in the prevention of age-related metabolic disorders and subsequent extended healthspan.

Although similar feeding conditions were performed, MCC950 treated mice showed less body weight gain. After short-term MCC950 treatment, metabolic profile was improved, showing better glucose response and higher levels of circulating adiponectin, which is directly correlated with improved insulin sensitibity [19]. Lower serum levels of leptin were also observed in MCC950-treated mice, what reflects smaller adipose tissue size and suggests that its secretion might be regulated by the NLRP3-inflammasome dependent proinflammatory

cytokines [20, 21], as well as a suitable ratio leptin: adiponectin. According to this, lipid accumulation in liver tissue was reduced after pharmacological inhibition of NLRP3, as well as fibrotic fibers. Liver size, total cholesterol and triglyceride levels resulted significantly diminished. All these observations on the protective effect of MCC950 against liver injury onset in aged mice are in concordance with a novel study performed in obese mice where this molecule enhanced NAFLD pathology [10]. Moreover, microarray data showed interesting data of upregulated genes improving lipid metabolism and inflammation [7] which supports the abovementioned results. Taken these data together, MCC950 effects might be due to the blockade of cholesterol crystals activating NLRP3-inflammasome in myeloid cells.

There is abundance evidence on mainly four model organisms sharing conserved genes that establish complex interrelationships among them involved in aging and lifespan. From yeast to mammals, all of the aforementioned organisms showed extended lifespan when mTOR and mTOR upregulators were attenuated and AMPK, however remained active [22-27], which apart from contributing to extend lifespan and healthy aging, its chronic activation has shown to decrease adiposity in liver tissue [27]. Taking this into account, NLRP3-inflammasome showed to be involved in longevity network regulation, since after using the inhibitor, the expression of several genes playing crucial roles in life and healthspan, including mTOR, PI3K, AKT, were significantly decreased, whereas AMPK was overexpressed in the livers of MCC950-treated group. These experiments were reproduced in vitro, where we observed that NLRP3 could somehow regulate mTOR pathway and viceversa, resulting in healthier aging promotion.

Autophagy is a main cytoprotective mechanism contributing to cellular homeostasis whose dysfunction is associated with a variety of

pathologies including heart and liver disease. As we age, authophagic machinery becomes impaired and less effective, causing defective clearance and therefore, contributing to tissue damage [28]. Autophagy and autophagy-related proteins are vital to modulate inflammatory response, since autophagy is connected to innate immune receptors ensuring the intracellular degradation of microbial presence or ROS. Besides, the activation of autophagy by inflammatory signals limits proinflammatory cytokines production by targeting inflammasomes to ensure their elimination [29]. According to this, our results indicate that MCC950 inflammasome inhibitor, contributes to ameliorate autophagic flux.

Notably, enhanced healthspan in mice treated with MCC950 is associated with amelioration of lipid and glucose metabolism, autophagy and the regulation of genes involved in longevity pathways. Moreover, MCC950 seemed to upregulate PPARα, which contributes to the abovementioned improved metabolic profile. Although more research should be performed in this field to provide more data of how MCC950 could affect other tissues, our data demonstrate that pharmacological inhibition of NLRP3-inflammasome extends healthspan by preventing old mice from aged-related metabolic disabilities, including better glucose assimilation, less insulin resistance as well as ameliorated autophagic flux and the regulation of important genes involved in longevity pathways.

CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, our data suggests that MCC950 molecule shows beneficial metabolic and inflammatory effects, as well as ameliorated autophagic machinery in aged mice liver tissues.

Pharmacological inhibition of NLRP3-inflammasome may hold a promising therapy to extend healthspan by enhancing age-related metabolic disabilities. Based on our findings, future studies will be required to test the hypothesis that the inhibition of NLRP3-inflammasome with the specific molecule MCC950 extends healthspan in aged individuals.

Acknoledgements

This study was supported by a grant from the Andalusian regional government (Grupo de Investigacion Junta de Andalucia CTS113), Consejería de Salud de la Junta de Andalucia: PI-0036-2014. F.M. has the benefit of a FPU fellowship (FPU13/03172) from the MECD.

REFERENCES

1. Angulo P. Nonalcoholic fatty liver disease. *N Eng J Med.* **2002**, 346: 1221-1231.

2. Ip E, Farrell GC, Robertson G, *et al.* Central role of PPARalpha-dependent hepatic lipid turnover in dietary steatohepatitis in mice. *Hepathology.* **2003**, 38: 123-132.

3. Ip E, Farrell GC, Hall P, *et al*. Administration of the potent PPARalpha agonist, Wt-14,643, reverses nutritional fibrosis and steatohepatitis in mice. *Hepathology.* **2004**, 39: 1286-1296.

4. Francque S, Verrijken A, Caron S, *et al.* PPARα gene expression correlates with severity and histological treatment response in patients with non-alcoholic steatohepatitis. *J Hepatol*. **2015**, 63: 164-173.

5. Tailleux A, Wouters K, Staels B. Roles of PPARs in NAFLD: Potential therapeutic targets. *Biochim Biophys Acta*. **2012**, 5: 809-818.

6. Kallwitz ER, McLachlan A, Cotler SJ. Role of peroxisome proliferators-activated receptors in the pathogenesis and treatment of nonalcoholic fatty liver disease. *World J Gastroenterol.* **2008**, 14: 22-28.

7. Lefebvre P, Chinetti G, Fruchart JC, *et al*. Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis. *J Clin Invest.* **2006**, 116: 571-580.

8. Franceschi C, Bonafè M, Valensin S, *et al*. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci*. **2000**, 908: 244-254.

9. Coll RC, Robertson AA, Chae JJ, *et al*. A small molecule inhibitor of the NLRP3 –inflammasome for the treatment of inflammatory diseases. *Nat Med*. **2015**, 21: 248-255.

10. Mridha AR, Wree A, Robertson AAB, *et al*. NLRP3 inflammasome blockade reduces liver inflammation and fibrosis in experimental NASH in mice. *J Hepatol*. **2017**, 66: 1037-1046.

11. Pavillard LE, Cañadas-Lozano D, Alcocer-Gómez E, *et al*. NLRP3-inflammasome inhibition prevents high fat and high sugar diets-induced heart damage through autophagy induction. *Oncotarget*. **2017**, 21: 99740-99756.

12. Berg AH, Combs TP, Du X, *et al*. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med*. **2001**, 7:947-953.

13. Li Y, Xu S, Giles A, *et al*. Hepatic overexpression of SIRT1 in mice attenuates endoplasmic reticulum stress and insulin resistance in the liver. *FASEB*. **2011**, 25:1664-1679.

14. Kaeberlein M, Powers RW III, Steffen KK, *et al*. Regulation of yeast replicative life span by TOR and sch9 in response to nutrients. *Science*. **2005**, 310: 1193-1196.

15. Kapahi P, Zid BM, Harper T, *et al*. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr Biol*. **2004**, 14: 885-890.

16. Vellai T, Takacs- Vellai K, Zhang Y, *et al*. Genetics: influence of TOR kinase on lifespan in C Elegans. *Nature*. **2003**, 426: 620.

17. Barzilai N, Huffman DM, Muzumdar RH, *et al.* The critical role of metabolic pathways in aging. *Diabetes.* **2012**, 61: 1315-1322.

18. Youm YH, Grant RW, McCabe LR, *et al*. Canonical Nlrp3-inflammasome links systemic low-grade inflammation to functional decline in aging. *Cell Metab*. **2013**, 18: 519-532.

19. Kadowaki T, Yamauchi T, Kubota N, *et al.* Adiponectin and adiponectin receptors in insulin resistance, diabetes and the metabolic syndrome. *J Clin Invest*. **2006**, 116: 1784-1792.

20. Frederich RC, Hamann A, Anderson S, *et al.* Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med*. **1995**, 1: 1311-1314.

21. Dagogo-Jack S. Human leptin regulation and promise in pharmacotherapy. *Curr Drug Targets*. **2001**, 2: 181-195.

22. Johnson SC, Sangesland M, Kaeberlein M, *et al.* Modulating mTOR in aging and health. *Interdicip Top Gerontol*. **2015**, 40: 107-127.

23. Kaeberlein M, Powers RW 3rd, Steffen KK, *et al.* Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients.*Science.* **2005**, 310 :1193-1196.

24. Kapahi P, Zid BM, Harper T, *et al.* Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr Biol.* **2004**, 14: 885-890.

25. Vellai T, Takacs-Vellai K, Zhang Y, *et al*. Genetics: influence of TOR kinase on lifespan in C. elegans. *Nature*. **2003**, 426: 620.

26. Martin-Montalvo A, Mercken EM, Mitchell SJ, *et al*. Metformin improves healthspan and lifespan in mice. *Nat Commun*. **2013**, 4: 2192.

27. Yang J, Maika S, Craddock L, *et al*. Chronic activation of AMP-activated protein kinase-alpha1 in liver leads to decreased adiposity in mice. *Biochem Biophys Res Commun*. **2008**, 370: 248-53.

28. Linton PJ, Gurney M, Sengstock D, *et al.* This old heart: Cardiac aging and autophagy. *J Mol Cell Cardiol.* **2015**, 83: 44-54.

29. Nakahira K, Haspel JA, Rathinam VA, *et al.* Autophagy proteins regulate innate immune respnses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol. 2011, 12: 222-230.

SUPPLEMENTAL MATERIAL

Supplementary methods

Thp1 experiments

THP-1 cells, a human leukemia monocytic cell line extensively used to study monocyte/macrophage functions, were cultured at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with L-glutamine, an antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO, USA), and 10% fetal bovine serum.

To induce NLRP3-activation, cells were expose to LPS (250 ng/ml, 4h) for priming, and to ATP (5mM, 24h) for triggering. For protein synthesis experiment, cells were treated with puromycin (1μM) and harvested after 30 minutes.

Serum biomarkers

Serum levels of cholesterol, triglycerides, albumin, bilirubin, alanine and aspartate aminotransferases, lactate dehydrogenase, creatine phosphokinase, uric acid and creatinine were assayed using commercial kits (Randox Laboratories, Antrim, UK).

QRT-PCR analysis

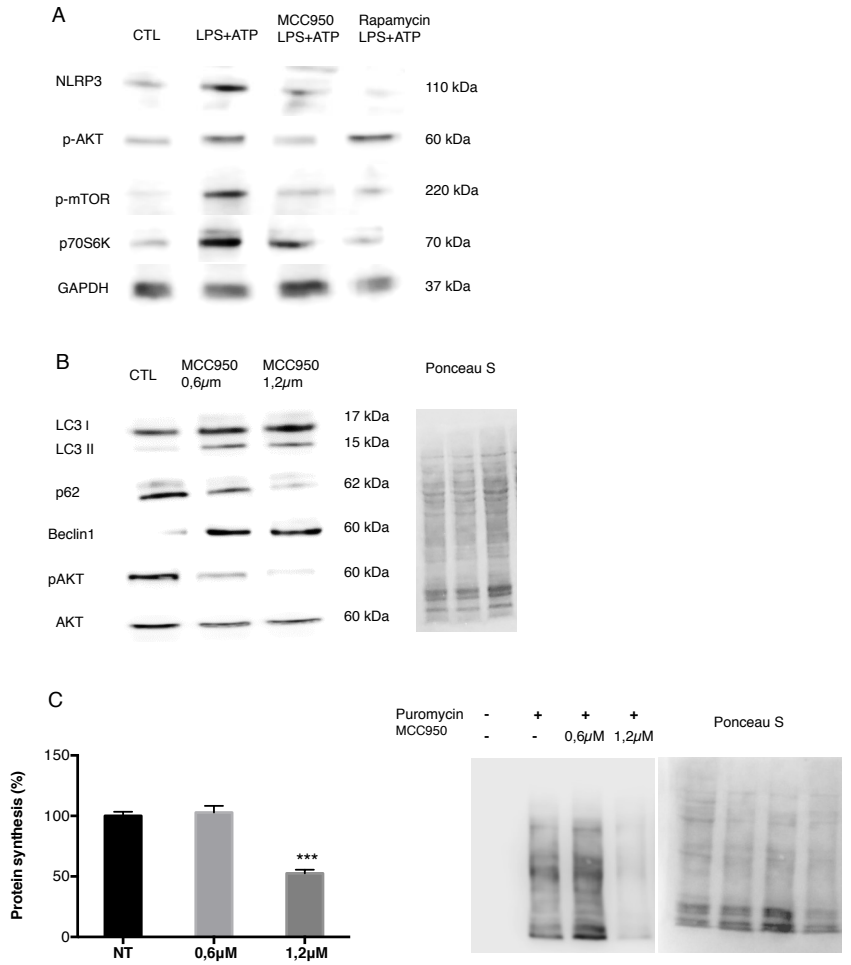
Total RNA from mouse liver was extracted using RNeasy Tissue Mini Kit (QIAGEN) and treated with DNase. CDNA was synthesized using iScript (BioRad) cDNA kit. QRT-PCR was performed on the ROCHE LightCycler® 96 Instrument with SYBR Green Master Mix (BioRad) using 50 ng of cDNA per reaction with 3x reactions/sample. ß-actin was used as housekeeping gene. All primers

were designed based on Primer 3plus and can be found in Supplementary Table 4.

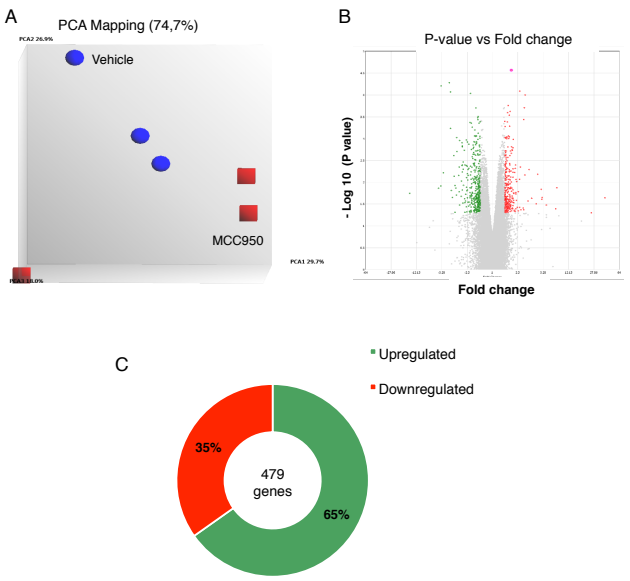
Protein synthesis

Briefly, the cells were incubated with serum-free DMEM for 90 min followed by incubation with 1 μM puromycin (an analogue of tyrosyl-tRNA; Invitrogen; A11138-03) for 30 min. The amount of puromycin incorporated into nascent peptides was then evaluated by Western blot.

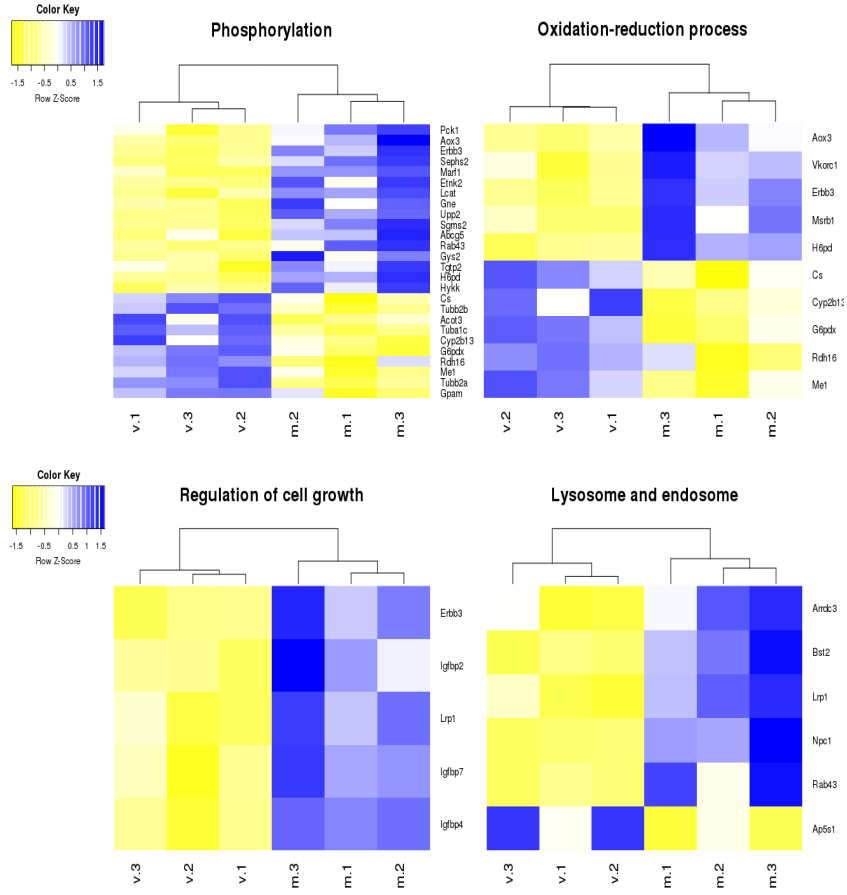
Supplemental figures



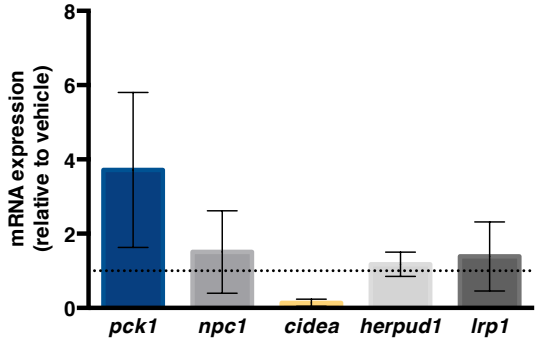
Supplemental figure 1. NLRP3-inflammasome might be involved in autophagy and protein synthesis by regulating TOR pathway and viceversa. (A) Western Blot images showing NLRP3 and TOR pathway, using NLRP3 activators, such as LPS and ATP and NLRP3 and mTOR inhibitors, MCC950 and rapamycin, respectively. (B) Western Blot analaysis showing autophagy markers: LC3, p62, Beclin1, pAKT, AKT, (C) Protein synthesis was quantified in protein extracts treated with MCC950 using puromycin labeling followed by immunoloblot. NT: No treatment.



Supplemental figure 2. Inhibition of NLRP3 protects age-related liver disorders in transcriptome. (A) Principal component analysis (PCA) was performed in differentially expressed genes from the liver of mice treated with the vehicle or MCC950. Each data point corresponds to the PCA analysis of each subject, (B) Volcano plots of gene expression difference in liver in vehicle and MCC950-treated old mice. Grey color means gene probes that do not reach cuto-ff levels for statistical significance as well as fold change. Green color represents genes with decreased expression in aged MCC950-treated mice when compared to aged vehicle; whilst, red color marks genes with increased expression in MCC950-treated mice, (C) Representation of the 479 genes that reached the cut-off, of which, 65% were upregulated and 10% were downregulated on liver tissue from vechicle and MCC950-traeted mice.



Supplemental figure 3. Microarray data. Effect of MCC950 on phosphorylation, oxidation-reduction processes, regulation of cell growth and lysosomal and endosomal regulation during aging.



Supplemental figure 4. qRT-PCR Validation. qRT-PCR Validation of various up- and down-regulated genes from microarray analaysis.

Supplemental tables

Supplemental table 1. Effects of MCC950 treatment on various serum biomarkers.

Parameters	Old vehicle	Old MCC950
Cholesterol (mg/dL)	310.9 (15.2)	190.1 (17.4)***
Triglycerides (mg/dL)	73.81 (13.4)	69.15 (16.1)
Albumin (mg/dL)	2.31 (0.12)	2.02 (0.11)
Bilirubin (mg/dL)	0.16 (0.01)	0.14 (0.02)
Ala aminotransferase (UL)	499.8 (71)	315.37 (85)**
Asp aminotransferase (UL)	471.8 (83)	364.8 (97)**
Lactate dehydrogenase (UL)	1499 (198)	987 (211)***
Creatine phosphokinase (UL)	6190 (998)	4127 (691)***
Uric Acid (μMol/dL)	28.15 (4.3)	26.31 (6.1)
Creatinine (mg/dL)	0.65 (0.05)	0.62 (0.03)

Values are presented as mean ± SEM. UL, units per litre. *$P<0.05$, **$P<0.005$, ***$P<0.001$ versus vehicle. (n=10).

Supplemental table 2. Microarray data. Genes significantly upregulated after MCC950-treatment. (FDR<0.05, more than 1.5 fold).

Gene Symbol	Fold change (linear)	ANOVA p-value	FDR p-value	Accession number
Pck1	5,82	0,04182	0,97452	NM_011044
Herpud1	5,38	0,000808	0,820725	NM_022331
Saa4	4,12	0,000686	0,820725	NM_011316
Arrdc3	3,84	0,044751	0,97452	NM_001042591
Angptl4	3,51	0,025994	0,97452	NM_020581
Egr1	3,32	0,040501	0,97452	NM_007913
Selenbp2	3,23	0,005968	0,97452	NM_019414
Cyp2d13	3,2	0,009951	0,97452	NR_003552
Rgs16	2,99	0,047614	0,97452	NM_011267
Serpina3n	2,68	0,007576	0,97452	NM_009252
Selenbp1	2,67	0,008072	0,97452	NM_009150
Bst2	2,35	0,005907	0,97452	NM_198095
Sdc4	2,35	0,016673	0,97452	NM_011521
Rplp1	2,33	0,038841	0,97452	NM_018853
Aox3	2,31	0,045074	0,97452	NM_023617
Fbxo21	2,28	0,022739	0,97452	NM_145564
Nr0b2	2,25	0,029354	0,97452	NM_011850
Slc25a47	2,21	0,024702	0,97452	NM_001012310
Lims2	2,1	0,002503	0,97452	NM_144862
Slc17a2	2,1	0,046818	0,97452	NM_144836
F11	2,06	0,024048	0,97452	NM_028066
Dbp	2,01	0,038376	0,97452	NM_016974
Slc8b1	2	0,002515	0,97452	NM_001177594
Ifi27	2	0,008159	0,97452	NM_026790
Vkorc1	1,98	0,038492	0,97452	NM_178600
Il6ra	1,93	0,003168	0,97452	NM_010559
Erbb3	1,88	0,006558	0,97452	NM_010153
Igfbp2	1,85	0,028556	0,97452	NM_008342
Rps27rt	1,84	0,00542	0,97452	ENSMUST00000053150
Sephs2	1,81	0,008951	0,97452	NM_009266
Marf1	1,8	0,003662	0,97452	NM_001081154
Zfp36l2	1,78	0,010715	0,97452	NM_001001806
Erdr1	1,77	0,02906	0,97452	NM_133362
Cep85l	1,77	0,047017	0,97452	NM_001204983
Etnk2	1,76	0,028711	0,97452	NM_175443
Lrp1	1,74	0,010182	0,97452	NM_008512
Lcat	1,73	0,004131	0,97452	NM_008490
Ighv7-3	1,73	0,005503	0,97452	ENSMUST00000103461
Mir466f-2	1,72	0,026186	0,97452	ENSMUST00000104805
Rps27rt	1,71	0,037421	0,97452	NM_001190258
Mrip-ps	1,7	0,016848	0,97452	OTTMUST00000117311
Npc1	1,69	0,005546	0,97452	NM_008720
Vmn1r39	1,68	0,014043	0,97452	NM_001166720
Gne	1,68	0,021982	0,97452	NM_001190414
Avpr1a	1,67	0,02639	0,97452	NM_016847
n-R5s153	1,64	0,022413	0,97452	ENSMUST00000083451
Slc7a2	1,64	0,046524	0,97452	NM_001044740
Upp2	1,63	0,0009	0,820725	NM_001289659
Sgms2	1,63	0,00814	0,97452	NM_028943

Cyp2d41-ps	1,63	0,043645	0,97452	OTTMUST00000112044
Dnajc3	1,63	0,046726	0,97452	NM_008929
Ifit1	1,62	0,008412	0,97452	NM_008331
Tmem50a	1,61	0,000828	0,820725	NM_027935
Olfr846	1,61	0,048127	0,97452	NM_146282
C6	1,6	0,038104	0,97452	NM_016704
Mafb	1,59	0,007077	0,97452	NM_010658
Trp53inp1	1,57	0,037755	0,97452	NM_001199105
Masp1	1,57	0,043801	0,97452	NM_008555
Abcg5	1,56	0,034109	0,97452	NM_031884
Mir3966	1,55	0,026528	0,97452	NR_039547
Rab43	1,55	0,027935	0,97452	NM_001039394
Gys2	1,55	0,034804	0,97452	NM_145572
Zbtb16	1,55	0,037067	0,97452	NM_001033324
C1rb	1,55	0,040777	0,97452	NM_001113356
F12	1,55	0,048214	0,97452	NM_021489
Il1r1	1,54	0,014788	0,97452	NM_001123382
Maf	1,54	0,023496	0,97452	NM_001025577
Msrb1	1,54	0,028941	0,97452	NM_013759
Cmtm8	1,54	0,029956	0,97452	NM_027294
Tgtp2	1,53	0,04922	0,97452	NM_001145164
H6pd	1,52	0,005755	0,97452	NM_173371
Igfbp7	1,52	0,010924	0,97452	NM_001159518
Igkv2-109	1,52	0,027246	0,97452	NCBI_Gene:628268
Olfr821	1,52	0,038342	0,97452	NM_146776
Scarb1	1,51	0,003537	0,97452	NM_001205082

Values of fold change (linear), ANOVA p-value, and FDR p-value are represented as MCC950 vs vehicle.

Supplemental table 3. Microarray data. Genes significantly downregulated after MCC950-treatment. (FDR<0.05, more than 1.5 fold).

Gene Symbol	Fold change (linear)	ANOVA p-value	FDR p-value	Accession number
Gpam	-4,97	0,043305	0,97452	NM_008149
Tubb2a	-2,92	0,001043	0,820725	NM_009450
Me1	-2,9	0,024593	0,97452	NM_001198933
Cidea	-2,78	0,002715	0,97452	NM_007702
Cd36	-2,44	0,000698	0,820725	NM_001159555
Lgals1	-2,43	0,028547	0,97452	NM_008495
Mir1958	-2,15	0,047763	0,97452	NR_035484
Mfsd2a	-2,1	0,017813	0,97452	NM_029662
Snord90	-2,08	0,021007	0,97452	NR_028535
Rdh16	-2	0,046762	0,97452	NM_009040
Onecut1	-1,97	0,02037	0,97452	NM_008262
Vmn1r204	-1,87	0,006055	0,97452	NM_001045544
Klhl34	-1,79	0,029266	0,97452	NM_001081667
Tcrg-V6	-1,79	0,034813	0,97452	NCBI_Gene:21640
Olfr101	-1,78	0,001039	0,820725	NM_146834
G6pdx	-1,75	0,017411	0,97452	NM_008062
Ift88os	-1,73	0,020171	0,97452	ENSMUST00000149702
Xlr3c	-1,71	0,02238	0,97452	NM_011727
Cyp2b13	-1,71	0,035242	0,97452	NM_007813
Tuba1c	-1,69	0,003109	0,97452	NM_009448
Scgb2b19	-1,68	0,000418	0,820725	NM_001199336
Acot3	-1,67	0,03839	0,97452	NM_134246
Mir6910	-1,66	0,00532	0,97452	NR_105875
Igkv4-61	-1,65	0,035479	0,97452	NCBI_Gene:546244
Ap5s1	-1,64	0,044448	0,97452	NM_027129
Hist1h4f	-1,64	0,045277	0,97452	NM_175655
Mir6412	-1,63	0,000959	0,820725	ENSMUST00000184477
Igkv4-58	-1,59	0,023294	0,97452	NCBI_Gene:381831
Tubb2b	-1,57	0,010945	0,97452	NM_023716
Igkv4-81	-1,56	0,035199	0,97452	NCBI_Gene:243453
Vmn2r-ps19	-1,55	0,026644	0,97452	OTTMUST00000111832
Tas2r126	-1,54	0,018614	0,97452	NM_207028
Msl3l2	-1,53	0,023084	0,97452	NM_001163833

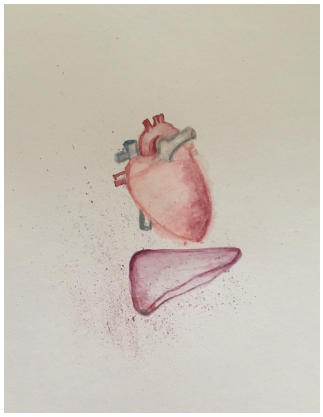
Mrap	-1,52	0,002825	0,97452	NM_029844
Olfr734	-1,52	0,00685	0,97452	NM_146664
Mrps23	-1,52	0,018428	0,97452	NM_024174
Traj25	-1,52	0,038061	0,97452	NCBI_Gene:100124364
Cs	-1,52	0,04376	0,97452	NM_026444
Olfr779	-1,51	0,00109	0,820725	ENSMUST00000069063
Tubb4b-ps1	-1,51	0,017976	0,97452	ENSMUST00000179460

Values of fold change (linear), ANOVA p-value, and FDR p-value are represented as MCC950 vs vehicle.

Supplemental table 4. Primer pairs used for real-time-PCR analyses for microarray validation.

Target	Accesion Number	Direction	Sequence (5'-3')
Pck1	NM_011044.2	Forward	GTCAACACCGACCTCCCTTA
		Reverse	CATTGTGCCGCTATCTCAAA
Npc1	NM_008720.2	Forward	ACCAGCAGTGTTGGGATAGG
		Reverse	CATGTGTTGGGACTCCACAG
Herpud1	NM_022331.1	Forward	ACAAAGGGTGCTGAATCCAC
		Reverse	CCTTGGAAAGTCTGCTGGAC
Cidea	NM_007702.2	Forward	GCAGCCTGCAGGAACTTATC
		Reverse	CCATTTCTGTCCCTTTTCCA
Lrp1	NM_008512.2	Forward	CTGAAGGCTCCGAGTACCAG
		Reverse	GTAGGAGATTGTGCCCGTGT
ß-actin	NM_007393.5	Forward	TGTTACCAACTGGGACGACA
		Reverse	GGGGTGTTGAAGGTCTCAAA

CHAPTER 04



General discussion

&

Conclusions

GENERAL DISCUSSION

Aging is the greatest risk factor for the development of cardio-metabolic events. Genetics, inactivity, overnutritition and, in general, unhealthy behavioural habits are main contributors to chronic low-grade inflammatory state, triggering aged-related diseases [1].

Both NLRP3-inflammasome deletion and its inhibiton with MCC950 showed progressive decrease in body weight gain and almost unaltered food intake during the entire observation periods. Better glucose response, insulin sensitivity and adiponectin serum levels were dectected in the absence of the inflammasome NLRP3, what demonstrated an enhancement in cardio-metabolic parameters. Moreover, reduced levels of circulating glucose and IGF-1 have been reported to be indicatives of stress resistance and are linked to antiaging effects [2]. Adipokines such as leptin and adiponectin, and the leptin/adiponectin ratio, are highly linked to CVD, metabolic syndrome, type 2 diabetes mellitus and NAFLD [3]. Both NLRP3-inflammasome deletion and MCC950-treated mice showed reduced leptin and leptin/adiponectin ratio levels as well as an increase in circulating adiponectin.

Furthermore, some interesting genes resulted up- or downregulated by performing microarray profiling in MCC950-treated liver tissue. *Npc1, pck1, herpud1* and *lpr1*, which are involved in metabolic response, including cellular cholesterol content [4], increased gluconeogenesis, adequate glucose uptake [5] and hepatic insulin resistance [6] respectively, resulted upregulated. However, *cidea* was downregulated, which is associated with less severity of liver steatosis [7] and in concordance with previous metabolic results. Interestingly, overexpressed PPAR family genes were pretty close to the cut-off

stablished (1.4-fold change), what indicated once more, better lipid metabolism both in vivo and in vitro.

Taking these data together, NLRP3-inflammasome inhibition or deletion has shown to enhance cardio-metabolic homeostasis during the aging process.

It is widely acknowledged that some changes take place at cardiac level as we age. Even healthy aged individuals experiment heart weight gain and slight age-associated ECG variations. The accumulation of lipid content, such as lipofucsin and fibrotic tissue, are common aspects of the elderly as well. However, old NLRP3-/- mice showed less incidence of fibrosis, less LV occlusion and decreased heart size. All these changes were associated with animals less prone to atrial fibrillation and therefore, minor risk to develop heart failure [8, 9], which may correlate with healthier aging and extended healthspan through cardiac function improvement.

Apart from heart alterations and changes that aging triggers, morphological variations in liver do occur as part of physiological aging even in healthy individuals. Liver steatosis and fibrotic tissue as well as total cholesterol and TGs levels in liver resulted reduced after MCC950 treatment. Moreover, hepatic reduction of liver weight and reduced visual tumor incidence inspection were associated with MCC950 metabolic effects.

The scientific community has made numerous endeavours to further understand how cardio-metabolic events take place and develop during aging. Cardiac and liver homeostasis is partly regulated by the kinase mTOR. The mTOR pathway has been largely studied because of its involvement in longevity, and given the observed low levels of IGF-1

in the absence of NLRP3-inflammasome in old mice, and the already existing evidence reporting that mTOR partial attenuation is beneficial for cell survival and lifespan [10], assays on mTOR pathway signalling were considered. It has been reported that mTOR activation is related to cardiac hypertrophy and genetic complications at cardiac level [11, 12]. Interestingly, NLRP3-/- mice presented parcial inhibition of mTOR in the heart since early ages, what significantly protected this group from cardiac decline during aging.

A similar situation was observed in liver. Reduced protein expression levels of phosphorylated PI3K, AKT and mTOR and increased levels of phospho-AMPK in old mice after MCC950-treatment were detected. AMPK and AKT are regulators of mTOR. The attenuated hepatic mTOR levels were therefore, due to increased expresion of p-AMPK and reduced levels of p-AKT in liver tissue as well.

Attenuated pmTOR expression in heart and liver tissue of NLRP3-inflammasome KO mice and MCC950-treated WT mice contributed to autophagic machinery activation [13, 14]. Autophagy is the main cytoprotective mechanism involved in cellular homeostasis whose impairment is highly associated with cardio-metabolic disabilities. It is already known, that autophagy process becomes less effective with aging, contributing to cardiac and liver damage [15]. Cardiac decline during aging triggers stress which, as a strategy of cell survival activates autophagy machinery. NLRP3-/- mice presented rapid autophagy and great autophagic flux maintained over time, which exerted cardioprotective effects in old NLRP3-/- group.

In liver, ameliorated autophagic flux was detected as well. Autophagy-related proteins regulate innate immune response inflammatory responses by ensuring intracellular degradation of PAMPs

and DAMPs [16]. When inflammatory signals activate autophagy, the production of proinflammatory cytokines gets limited by targeting the inflammasomes. Besides, it has been recently reported that MCC950 treatment in mice induced autophagy and improved autophagic flux after high energy-content diet [14], what is in concordance with our data obtained in liver tissue. In vitro experiments in Thp1 cells showed similar autophagic profile when treated either with MCC950 or rapamycin. Surprisingly, MCC950's effect is attributable to the inhibition of mTOR pathway shortly after ATP and LPS treatment to activate NLRP3-inflammasome. Liver environment was reproduced in vitro using HepG2 cells treated with MCC950, which resulted in induced autophagy and a reduction of protein synthesis. A characteristic effect when mTOR is partially inhibited.

Accordingly, MCC950 effects are in concordance with previous studies regarding NLRP3-inflammasome deletion, where animals exerted better glycemic control, ameliorated cognitive function as well as attenuated bone loss [17]. Moreover, this is also consistent with data obtained in **chapter 02**, where NLRP3 genetic ablation enhanced cardiac function in young and old NLRP3-/-mice.

Although genetic modifications are a great strategy to target age-related cardiometabolic events since early ages, pharmacological intervention should be taken into account as an interesting approach to extend healthspan in humanbeing.

GENERAL CONCLUSIONS

From this Thesis it can be cocluded that:

1. NLRP3-inflammasome genetic deletion ameliorates cardio-metabolic homeostasis by improving glucose response, insulin resistance, and leptin/adiponectin ratio when compared with WT mice of the same age.

2. NLRP3-inflammasome deletion extends medium lifespan.

3. NLRP3-inflammasome deletion delays cardiac aging by exerting cardioprotective effects.

4. NLRP3-inflammasome deletion induces mTOR attenuation, which results in improved autophagy machinery and autophagic flux.

5. Pharmacological specific inhibition with MCC950 reproduced similar effects in aged treated mice, amelioranting metabolic liver profile and age-related changes in liver transcriptome.

6. MCC950-treated old mice presented enhanced autophagy as well as less expression of mTOR pathway signalling proteins.

7. Both NLRP3-inflammasome genetic modification and MCC950 pharmacological inhibition contributed to general improvement of cardiac and liver function in the elderly, promoting healthy aging and extended healthspan when compared with WT or vehicle-treated animals of the same age.

REFERENCES

1. Pavillard LE[1], Marín-Aguilar F[1], Bullón P, Cordero MD. Cardiovascular disease, NLRP3-inflammasome, and western dietary patterns. *Pharmacol Res.* **2018**, 131: 44-50.

2. Brandhorst S, Choi IY, Wei M, *et al.* A periodic diet that mimics fasting promotes multi-system regeneration, enhanced cognitive performance, and healthspan. *Cell Metab.* **2015**, 22: 86-99.

3. DiNicolantonio JJ, Lucan SC, O'Keefe JH. The evidence for saturated fat and for sugar related to coronary heart disease. *Prog Cardiovasc Dis.* **2016**, 58: 464-472.

4. Ma X, Hu YW, Mo ZC, *et al.* NO-1886 up-regulates Nieman-Pick C1 protein (NPC1) expression through liver X receptor alpha signalling pathway in THP-1 macrohage-derived foam cells. *Cardiovasc Drugs Ther.* **2009**, 23: 199-206.

5. Navarro-Marquez M, Torrealba N, Troncoso R, *et al.* Herpud1 impacts insulin-dependent glucose uptake in skeletal muscle cells by controlling the Ca2+-calcineurin-Akt axis. *Biochim Biophys Acta.* **2018**, 1864: 1653-1662.

6. Ding Y, Xian X, Holland WL, *et al.* Low-density lipoprotein receptor-related protein-1 protects against hepatic insulin resistance and hepatic steatosis. *EBiolmedicine.* **2016**, 7: 135-145.

7. Zhou L, Xu L, Ye J, *et al.* Cidea promotes hepatic steatosis by sensing dietary fatty acids. *Hepatology.* **2012**, 56: 95-107.

8. GoHeiijman J, Voigt N, Nattel S, *et al.* Cellular and molecular electrophysiology of atrial fibrillation initiation, maintenance, and progression. *Circ Res.* **2014**, 114: 1483-1499.

9. Rocket DC, Bell PD, Hill JA. Fibrosis- a common pathway to organ injury and failure. *N Eng J Med.* **2015**, 372: 1138-1149.

10. Wu JJ, Liu J, Chen EB, *et al.* Increased mammalian lifespan and a segmental and tissue-specific slowing of aging after genetic reduction of mTOR expression.

11. McMullen JR, Sherwood MC, Tarnavski O, *et al*. Inhibition of mtor signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. *Circulation*. **2004**, 109: 3050–3055.

12. Marin TM, Keith K, Davies B, *et al*. Rapamycin reverses hypertrophic cardiomyopathy in a mouse model of leopard syndrome-associated ptpn11 mutation. *J Clin Invest*. **2011**,121:1026–1043.

13. Wei YM, Li X, Xu M, *et al.* Enhancement of autophagy by simvastatin through inhibition of Rac1-mTOR signaling pthway in coronary arterial myocytes. *Cell Physiol Biochem.* **2013**, 31: 925-937. *Cell Rep.* **2013**, 4: 913-920.

14. Pavillard LE, Cañadas-Lozano D, Alcocer-Gómez E, *et al.* NLRP3-inflammasome inhibition prevents high fat and high sugar diets-induced heart damage through autophagy induction. *Oncotarget.* **2017**, 8: 99740-99756.

15. Linton PJ, Gurney M, Sengstock D, *et al.* This old heart: Cardiac aging and autophagy. *J Mol Cell Cardiol.* **2015**, 83: 44-54.

16. Nakahira K, Haspel JA, Rathinam VA, *et al.* Autophagy proteins regulate innate immune respnses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol. 2011, 12: 222-230.

17. Youm YH, Grant RW, McCabe LR, *et al.* Canonical NLRP3-inflammasome links systemic low-grade inflammation to functional decline in aging. *Cell Metab.* **2013**, 18: 519-532.

LIST OF ABBREVIATIONS

LIST OF ABBREVIATIONS

AD, Alzheimer's disease

AMPK, AMP-activated protein kinase

AKT, Serine/threonine kinase Akt

ALR, AIM2-like receptor

ASC, Apoptosis-associated speck-like containing a caspase domain

ATP, Adenosine triphosphate

CAPS, Cryopyrin-associated periodic fever syndromes

CKD, Chronic kidney disease

COPD, Chronic obstructive pulmonary disease

COX, Cytochrome C oxidase

CR, Calorie restriction

CVD, Cardiovascular disease

DAMPs, damage-associated molecular patterns

eNOS, Endothelial nitric oxide synthetase

ERK1/2, Extracellular signal-regulated kinase

GBP5, Guanylate-binding protein 5

Gsdmd, Gasdermin D

IGF-1, Insulin/insulin-like growth factor 1

IL, Interleukin

IFN, Interferon

IFNAR, Interferon alpha/beta receptor

IRF, Interferon regulatory transcription factor

JAK/STAT, Janus kinase/signal transducer and activator of transcription

LC3, Microtubule associated protein 1 light chain 3

LRR, Leucin-rich repeat domain

LPS, Lipopolysaccharide

MD, Macular degeneration

MetS, Metabolic syndrome

mtDNA, Mitochondrial DNA

mTOR, Mammalian target of rapamycin

NAD, Nicotinamide adenine dinucleotide

NAFLD, Non-alcoholic fatty liver disease

Nampt, Nicotinamide phosphoribosyltransferase

NASH, Non-alcoholic steatohepatitis

Nek7, NIMA-related kinase 7

NF-kß, Nuclear factor kappa beta

NO, Nitric oxide

NLR, Nod-like receptor

OST, Oxidative stress theory

PAMPs, Pathogen-associated molecular patterns

PARP-1, Poly (ADP-ribose) polymerase-1

PBMCs, Peripheral blood mononuclear cells

PD, Parkinson's disease

PKR, RNA-dependent protein kinase

PPARs, Peroxisome proliferator-activated receptors

ROS, Reactive oxygen species

SASP, Senescence-associated secretory phenotype

TLR4, Toll-like receptor 4

TRIF, Tir domain containing-adapter-inducing interferon beta

T2DM, Type 2 diabetes mellitus

ULK1, Unc-51 like kinase-1

WHO, World Health Organization

This is a list of most used abbreviations, but we have decided to spell out each abbreviation the first time it is used in each chapter, followed by the abbreviation in parentheses.

CURRICULUM VITAE

FABIOLA MARÍN AGUILAR

1. Personal Information

Date and place of birth: May 6th, 1989 (Seville, Spain)

2. Education

PhD Fellowship (FPU13/03173)

Spanish Ministry of Education, Culture and Sports

September 2014- September 2018

Advisors: P Bullón and MD Cordero

Master in Pharmaceutical Care and Assistential Pharmacy

University of Seville- Seville, Spain

(September 2012- July 2013)

Bachelor of Science (BSc) in Pharmacy

University of Seville- Seville, Spain

(September 2007- July 2012)

Erasmus Fellowship

Universitè Paris Sud XI- Paris, France

(September 2010- July 2011)

3. Complementary Training

International workshop of inflammation and immunity: from bench to clinic. School of Pharmacy, Universidad de Sevilla- Sevilla, Spain (December 2017)

Course for the correct handling and animal welfare in experimental procedures (Certificate of functions A, B, C)
Centro de Formación Permanente. Universidad de Sevilla- Sevilla, Spain (March 2017)

Course in research tools for scientific research. School of Education. Universidad de Sevilla- Sevilla, Spain (October 2016)

Course in Plant extracts: Standards and Applications in dietetics and cosmetics. Universidad Alcalá de Henares- Madrid, Spain

4. Skills

4.1 Languages

Spanish	Native speaker
English	Cambridge Certificate in Advanced English (CAE) C1
French	Diplôme d'Études en Langue Française (DELF) B2

4.2 Knowledge areas

Areas: Aging, Cardiovascular aging, Metabolism, Autophagy, Inflammation, NLRP3-inflammasome

Cellular and Molecular Biology: Western Blot, qRT-PCR, Cell culture, ELISA

Statistical programs: GraphPad® Prism

5. Professional and Academic experience

5.1 Professional experience

PhD Fellowship (FPU13/03173)
Spanish Ministry of Education, Culture and Sports
September 2014- September 2018

Initiation to Research Scholarship
Career Initiation Scholarships Program for recent graduates
University of Seville- Seville, Spain
(July- September 2012)

Collaborative Grant from the Spanish Ministry of Education
University of Seville- Seville, Spain
(January- July 2011)

5.2 Academic experience

Teaching experience
Universidad de Sevilla
September 2014- May 2018

5.3 Stays abroad

Department of Biology at the **University of Rochester**
Vera Gorbunoba and Andrei Seluanov
Rochester, NY- United States of America- (31/05/2017- 30/11/2017)

6. Scientific activity

6.1 Publications

Book chapters

Bullón P, **Marín-Aguilar F**, Román-Malo L. AMP-activated Protein Kinase, AMPK/ Mitochondria in metabolic diseases. Vol 107, pp 129-152, 2016. ISSN 9783319435879. Springer International Publishing AG

Journal articles

1. **Fabiola Marín-Aguilar**, Beatriz Castejón-Vega, Elísabet Alcocer-Gómez, Macarena López-Sánchez, Avril A B Robertson, Matthew A Cooper, Eloisa Andújar-Pulido, Mónica Pérez-Alegre, Jordi Muntané, Antonio J Pérez-Pulido, Jesús Ruíz-Cabello, Pedro Bullón, Mario D Cordero. Inhibition of the NLRP3-inflammasome by MCC950 improves healthspan in aged mice. *Nat Commun*. Under review.

2. Pavillard LE*, **Marín-Aguilar F***, Bullon P, Cordero MD. Cardiovascular diseases, NLRP3 inflammasome, and western dietary patterns. *Pharmacol Res*. **2018**, 131:44-50

Pavillard LE, C Cañadas-Lozano D, Alcocer-Gómez E, **Marín-Aguilar F**, Pereira S, Robertson AAB, Muntané J, Ryffel B, Cooper MA, Quiles JL, Bullón P, Ruiz-Cabello J, Cordero MD. NLRP3-inflammasome inhibition prevents high fat and high sugar diets-induced heart damage through autophagy induction. *Oncotarget*. **2017,** 8:99740-99756.

3. **Marín-Aguilar F**, Pavillard LE, Giampieri F, Bullón P, Cordero MD. Adenosine Monophosphate (AMP)-Activated Protein Kinase: A New Target for Nutraceutical Compounds. *Int J Mol Sci*. **2017,** 18: 288.

4. Alcocer-Gómez E, Ulecia-Morón C, **Marín-Aguilar F**, Rybkjna T, Casas-Barquero N, Ruiz-Cabello J et al. Stress-Induced Depressive Behaviours

Require a Functional NLRP3 Inflammasome. *Mol Neurobiol*. **2016**, 53: 4874-42.

5. Bullón P, Alcocer-Gómez E, Carrión AM, Garrido-Maraver J, **Marín-Aguilar F**, Román-Malo L, et al. AMPK Phosphorylation Modulates Pain by Activation of NLRP3-inflammasome. *Antioxid redox signal*. **2016**, 24: 157-70.

6. Cordero MD, Alcocer-Gómez E, **Marín-Aguilar F**, Rybkjna T, Cotán D, Pérez-Pulido et al. Mutation in cytocrhome b gene of mitochondrial DNA in a family with fibromyalgia is associated with NLRP3-inflammasome activation. *J Med Genet*. **2016**, 53: 113-22.

7. Alcocer-Gómez E, Garrido-Maraver J, Bullón P, **Marín-Aguilar F**, Cotán, D, Carrión AM, et al. Metformin and caloric restriction induce AMPK-dependent restoration of mitochondrial dysfunction in fibroblast from Fibromyalgia patients. *Biochim Biophys Acta*. **2015**, 1852: 1257-67.

8. Sánchez-Domínguez B, Bullón P, Román-Malo L, **Marín-Aguilar F**, Alcocer-Gómez E, Cordero MD. Oxidative stress, mitochondrial dysfunction and, inflammation common events in skin of patients with Fibromyalgia. *Mitochondrion*. **2015**, 21: 69-75.

9. Bullón P, Román-Malo L, **Marín-Aguilar F**, Álvarez-Suárez JM, Giamperi F, Battino M, et al. Lipophilic antioxydants prevent lipopolisaccharide-induced mitochondrial dysfunction through mitochondrial biogenesis improvement. *Pharmacol Res*. **2015**, 91: 1-8.

11. Montserrat-de la Paz S*, **Marín-Aguilar F***, García-Giménez MD, Fernández-Arche MA. Hemp (Cannabis sativa L.) seed oil: analytical and phytochemical characterization of unsaponifiable fraction. *J Agric food Chem*. **2014,** 62: 1105-10.

12. Montserrat-de la Paz S, Ángel-Martín M, García-Giménez MD, **Marín-Aguilar F**, Fernández-Arche MA. Dietary supplementation evening primrose oil improves symptoms of fibromyalgia syndrome. *J Funct Foods*. **2012**, 5: 1279-87.

6.2 Patents

"Uso del aceite de cáñamo para la prevención y/o el tratamiento de la fibromialgia" **Use of hemp seed oil to prevent and/or treat fibromyalgia syndrome.** Universidad de Sevilla P201500071

6.3 Conference contribution and participation

58th European Congress of Geriatry and Gerontology

F. Marín Aguilar, D. Cañadas Lozano, E. Alcocer Gómez, L. Román Malo, P. Bullón, M.D. Cordero . Inflamasomme complex implication in aging metabolism. Hotel Barceló Renaciemiento, Sevilla, 2016 (Poster).

1st International Work Day in Health Sciences

Marín-Aguilar F, Román-Malo L, Cañadas-Lozano D, Martínez-Sahuquillo Rico A, Bullón P, Cordero MD. Mitochondrial metabolism evaluation after Nifedipine and Cyclosporine A treatment in an in vitro model. School of Medicine, University of Seville, 2015 (Poster).

7th Meeting of Young Pharmacologists in Andalucia

Marín-Aguilar F, Román-Malo L, Cañadas-Lozano D, Bullón P, Cordero MD. Hormetic effect of Nifedipine and Cyclosporine A on cell metabolism. School of Pharmacy, University of Granada, 2015 (Oral presentation).

28th ECNP Congress. European College of Neuropsychopharmacology

Alcocer-Gómez E, Casas-Barquero N, Marín-Aguilar F, Bullón P, Carrión AM, Sánchez-Alcázar JA, Cordero, MD. NLRP3-inflammasome complex is implicated in depressive behaviour induced by stress. Amsterdam, 2015 (Poster).

AGRADECIMIENTOS

AGRADECIMIENTOS

Parecía que este momento no iba a llegar, y sin embargo aquí me encuentro, escribiendo las últimas frases de lo que va a ser mi Tesis doctoral. Son muchas las personas que han seguido de cerca y contribuído a la finalización de este trabajo y por tanto muchas con las que me siento en deuda.

En primer lugar, quiero agraderle a mis directores de Tesis, Pedro Bullón y Mario David Cordero, su confianza plena en mi desde el primer momento. Hace ya 4 años y medio comenzábamos la andadura en este camino. Gracias por apoyarme en esta etapa, que supondrá sin duda un antes y un después en mi vida, y que siempre recordaré con inmenso cariño.

Al Ministerio de Educación, por brindarme la oportunidad de formarme durante este periodo y a todos los profesionales que de alguna manera contribuyeron a mi desarrollo profesional (y personal).

A mis compañeros de laboratorio: Elísabet, Beatriz, Diego, Ángel, Rafa, y a mi compañera de batallas y amiga Lourdes, (¡cuantísimos momentos!). A Lucía Maestre, mi psicológa particular, gracias por las charlas matutinas. A Vera Gorbunova y Andrei Seluanov y a su equipo de la Universidad de Rochester, por hacerme sentir como en casa durante 6 meses.

A mi madre, por enseñarme a ser valiente y a seguir adelante como única opción. Madre solo hay una. A mi padre, por ser un ejemplo de superación y por tener la habilidad de ser profesional y humilde al mismo tiempo. A mi tío Francisco, por su incondicional y desinteresada ayuda. A mis abuelas, quienes me enseñaron el valor del trabajo, la

verdadera Tesis de la vida. A mi hermano Víctor, por ser mi compañero de camino y de risas cómplices que nadie entiende, aunque nos separen miles de kilometros.

A mis amigos de siempre, y a los que se quedarán para siempre. A mis cuquis: Espe, Wisa y Ana. A Carmen, José Carlos, Juan, Gonzalo, María. Mi gran fortuna. A Sergio, quien me introdujo en el mundo científico. Por sus sabios consejos. A Juan, mi alter ego, mi media uva, mi buen humor. GRACIAS.

A todos los que quereis cambiar el Planeta. A los que creemos que sin ciencia no hay futuro. A ti, que se que me estarás viendo orgullosa desde donde estés. Todo esfuerzo recompensado es una Victoria.